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SHORT COMMUNICATION

Polymorphism of the endothelin-1 gene (rs5370) is a potential contributor to sickle cell disease pathophysiology

Kristen G. Navarro [a,c], Smith E. Agyingi [a,c], Chinedu K. Nwabuobi [b], Bolaji N. Thomas [a,*]

[a] Department of Biomedical Sciences, Rochester Institute of Technology, Rochester, NY, 14623, USA
[b] Department of Obstetrics and Gynecology, University of South Florida, Tampa, FL, USA

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Abstract Sickle cell disease has been shown to demonstrate extensive variability in disease severity among and between individuals, the variability highlighted by differing genetic haplotypes. Despite the abundance of reports of functional significance due to polymorphisms of endothelial nitric oxide synthase (eNOS) and endothelin-1 (ET-1) genes, the role of these polymorphisms in mediating sickle cell disease pathophysiology among African Americans is presently unclear. To deconvolute their potential significance among African Americans with sickle cell disease, we examined the genetic diversity and haplotype frequency of eNOS and ET-1 polymorphisms in disease (n = 331) and control (n = 379) groups, with a polymerase –chain reaction restriction fragment length polymorphism assay. We report that genotypic and allelic frequencies of eNOS variants are not significantly different between groups. eNOS homozygote mutants, which had been shown to have clinical significance elsewhere, showed no statistical significance in our study. On the other hand, and contrary to previous report among Africans with sickle cell disease, the endothelin-1 homozygous mutant variant showed significant difference in genotypic (p = 2.84E-12) and allelic frequencies (p = 2.20E-16) between groups. The most common haplotype is the combination of T786C homozygote wild-type variant with homozygote mutant variants of G5665T (ET-1) and Glu298Asp (eNOS). These results show that endothelin-1 (rs5370) polymorphism, rather than endothelial nitric oxide synthase polymorphism might play a significant role in disease severity or individual clinical outcomes among African Americans with sickle cell disease. This would have profound

* Corresponding author. Department of Biomedical Sciences, Rochester Institute of Technology, 153 Lomb Memorial Drive, Rochester, NY, 14623, USA. Fax: +1 585 475 5809.
E-mail addresses: kxn171@case.edu (K.G. Navarro), sea7534@rit.edu (S.E. Agyingi), cnwabuobi@health.usf.edu (C.K. Nwabuobi), bolaji.thomas@rit.edu (B.N. Thomas).
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[c] Contributed equally.

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implications for designing and/or advancing personalized care for sickle cell patients and relieving disease complications.

Introduction

Sickle cell disease, a red cell disorder with multiple acute and chronic complications, and differing pathophysiological outcomes, remains a healthcare challenge, especially in sub-Saharan Africa.[1–3] The greatest toll of disease in the United States is in the black community among African Americans and immigrant populations from sub-Saharan Africa, some parts of the Middle East and Caribbean with an African descent, in addition to cases and other closely-related red cell disorders from the Indian sub-continent.[4] Since sickle cell disease has a genetic basis, its pathophysiology (vaso-occlusive crises, stroke, leg ulceration, acute chest syndrome, pulmonary hypertension etc.) has been found to vary widely, at times following an interethnic pattern.[5–7] Disease manifestations have been shown to be regulated by polymorphisms of genes reported to display clinical significance, among which are the endothelial nitric oxide synthase (eNOS) (polymorphisms include one in the promoter region (T-786C), one in exon 7 (Glu298Asp), and the variable number of tandem repeats (VNTR) in intron 4) and endothelin-1 (ET-1) genes. The C-786 variant of eNOS T786C polymorphism (rs2070744) is a genetic risk factor for acute chest syndrome in female patients,[8,9] while eNOS VNTR in intron 4 has been associated with plasma nitric oxide levels and vasculopathy.[10] In fact, a study from India found an association between eNOS gene polymorphisms and sickle cell disease severity,[4] concluding that eNOS gene is a genetic modifier of phenotypic variation among patients, or a marker of prognostic value, based on association with SCD clinical outcome.[11] Endothelin-1 (rs5370) gene is another whose polymorphism has been implicated in disease pathophysiology, including progression of chronic glomerulosclerosis,[12] pulmonary hypertension[13,14] and vaso-occlusive episodes correlated with pain history.[15] Its G5665T and T8002C variants have been associated with abnormal vascular reactivity, while the C8002 allele appears to increase the risk of acute chest syndrome in sickle cell disease.[8]

Published reports from our group and others, have shown that there is a significant interethnic diversity in the distribution of eNOS variants[16–19] and this could be potential contributor to differing sickle cell pathophysiology.[19] Despite the reports from other populations,[4,5,8–11] we showed previously that there is no difference in the genotypic or allelic frequencies of eNOS and endothelin-1 variants or significance of both polymorphisms among sickle cell disease patients from Africa.[20] If this is the case, do endothelial nitric oxide synthase and endothelin-1 gene polymorphism contribute to clinical pathophysiology among phenotypically-related African Americans with sickle cell disease? In this report, we investigated the genetic diversity and haplotype frequency of eNOS and ET-1 gene polymorphisms among sickle cell patients, recruited as part of the National Institute of Health-funded Cooperative Study of Sickle Cell Disease (CSSCD). Control populations are African American self-identified individuals, recruited from Shreveport, LA, United States.

Materials and methods

This project was reviewed and approved by the Institutional Review Board, Rochester Institute of Technology, and is in accordance with Helsinki Declaration. Genomic DNA aliquots from African American sickle cell disease patients, as previously described[7,21] were graciously provided by Betty Pace (Augusta University, GA) while control DNA samples (non-sickle cell patients; recruited from Shreveport, LA) were provided by Joann Moulds (Grifols Inc). PCR genotyping for all three endothelial nitric oxide synthase (a polymorphism in the promoter region T786C, rs2070744; another in exon 7 Glu298Asp, rs1799983; and the variable number of tandem repeats in intron 4) and endothelin-1 (G5665T) polymorphisms were carried out as published.[19,20,22] Amplified PCR products were digested with specific restriction enzymes, as shown previously, while fragment size analysis was carried out as reported.[19,20,23]

Data was analyzed with a simple PERL script and conversion of original data files to an EH program format was carried out as described.[20] Differences in genotype and allele frequencies between disease and controls were assessed by odds ratio and chi square test. Haplotype frequencies were estimated and tested for disease differences with the EH program.[24] Tests for deviation from Hardy–Weinberg equilibrium were performed, with SNP's rejected based on the recommended threshold of $p < 0.05$ in control individuals.

Results and discussion

Sickle cell disease is an inheritable, single gene disorder, found between groups and a major contributor to significant childhood mortality, especially in sub-Saharan Africa, and has been reported to show significant interethnic variability in pathophysiology and disease severity. Previous reports have shown that endothelial nitric oxide synthase and endothelin-1 gene polymorphisms are significant in mediating or regulating sickle cell disease outcome or severity,[4–6,25] plus roles in other diseases or pharmacological applications.[26–30] Our previous work has shown that endothelial nitric oxide synthase and endothelin-1 gene polymorphisms have no significance among native Africans with sickle cell disease.[20]

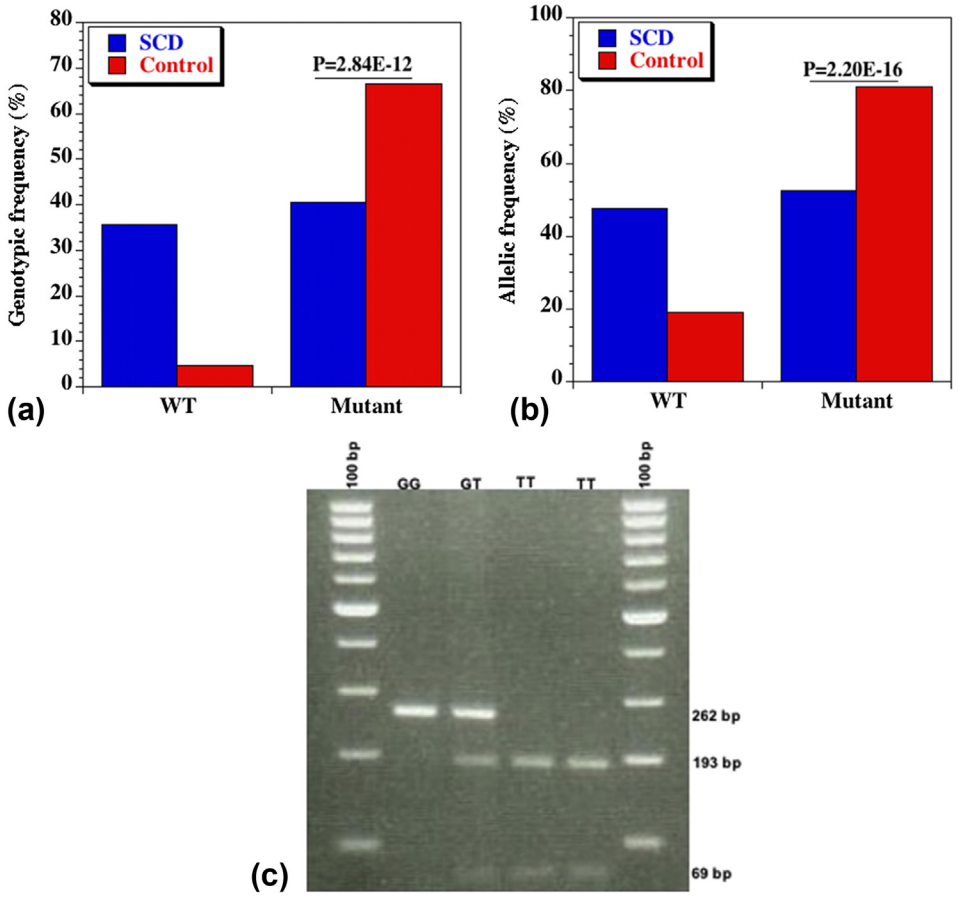

Fig. 1 Genotypic (a) and allelic (b) frequency of endothelin-1 G5665T (rs5370) gene polymorphism between sickle cell disease and control groups in United States. Two units of *Cac8I* restriction enzymes was used to digest amplified PCR products (c), yielding fragments of 262 base pair (homozygous wild type-GG) or 193 and 69 base pairs (homozygous mutant-TT). Blue bars-sickle cell disease patients; red bars-controls.

Table 1 Estimated eNOS and ET-1 gene haplotype frequencies between sickle cell disease and control groups.

Haplotypes			Haplotype frequencies		
T786C	Glu298Asp	G5665T	Case	Control	Case vs control
T	Glu	G	0.3364	0.1666	0.1682
T	Glu	T	0.4188	0.5727	0.2306
C	Glu	G	0.0794	0.0110	0.0446
C	Glu	T	0.0640	0.1213	0.0446
T	Asp	G	0.0479	0.0060	0.1028
T	Asp	T	0.0246	0.0682	0.3185
C	Asp	G	0.0120	0.0068	0.0081
C	Asp	T	0.0167	0.0473	0.0826

Abbreviations: SCD, sickle cell disease; CI, confidence interval; eNOS, endothelial nitric oxide synthase.
Odds ratio and *p*-value was calculated by two-tailed Fisher's exact test. Haplotype analysis was carried out with the EH program (lab.rockefeller.edu/ott/programs).

Following up on reports of clinical significance in other population groups, we evaluated the genotypic and allelic frequencies of endothelial nitric oxide synthase (eNOS) and endothelin-1 (ET-1) gene polymorphisms among and between African American sickle cell disease patients and controls and estimated the haplotype frequencies between groups. The polymorphisms analyzed are eNOS −786T>C (snp rs2070744) in the promoter region, the eNOS 298Glu>Asp (snp rs1799983) in exon 7, and the ET-1 5665G>T (snp rs5370) of the endothelin-1 gene. There was no statistically significant difference in the genotypic and allelic frequencies of eNOS variants between sickle cell disease and control groups. The homozygous wild type variants occurred at a higher and similar frequency in both groups (68.9% versus 64.4% for T/T (rs2070744) and 80.7% versus 75.5% for Glu/Glu (rs1799983) respectively). eNOS homozygous mutant variants are less frequent (3.3% versus 1.6% for rs2070744 and 0.9% versus 1.0% for rs1799983), and seem to lack any functional significance in sickle cell disease ($p = 0.15$ and 1 for eNOS −786T>C and eNOS 298Glu>Asp respectively), contrary to published reports elsewhere. A similar pattern was observed for allelic frequencies, with no difference between sickle cell and control groups ($p = 0.53$ and 0.13 for eNOS −786T>C and eNOS 298Glu>Asp respectively).

This current report shows the similarity of our previous observation on eNOS polymorphisms among and between Africans and African Americans.[19] Our data show that there

is no statistically significant difference in the genotypic or allelic frequencies of endothelial nitric oxide synthase polymorphisms between African American sickle cell disease and control groups. In addition, eNOS gene polymorphisms (rs2070744 and rs1799983) were equally distributed, with no deviations from the Hardy–Weinberg equilibrium. This lack of difference in eNOS gene polymorphisms between African and American sickle cell disease groups imply observations made in one population could potentially be applied to the other, with disease following a similar pattern on eNOS polymorphisms in both populations. Potential contribution of genomic heterogeneity in the control group[30] to our results would require further deconvolution, since significant admixture in this group could be a confounding factor to our observations and attributable conclusions. Following up with our previous report on the impact of geographical origin on genetic outcome among African American population,[19] further studies evaluating this polymorphism in representative groups, recruited from other parts of the United States vis-à-vis regional- or disease-specific selection pressures is imperative.

With the G5665T (rs5370) polymorphism on the ET-1 gene however, we found a statistically significant difference in both the genotypic ($p = 2.84E\text{-}12$) and allelic ($p = 2.20E\text{-}12$) frequencies of the mutant variant between sickle cell and control groups (Fig. 1). Comparing variants, we found the highest genotypic (40.5% versus 66.5%) and allelic (52.4% versus 80.9%) frequencies on the homozygous mutant loci (ET-1 5665T>T) for both sickle cell disease and control groups, contrary to previous observation among Africans with sickle cell disease.[20] That the highest frequency for the G5665T (rs5370) homozygous mutant variant was found among controls and not sickle cell disease patients, calls into question previous reports of endothelin-1 polymorphisms, as being implicated in sickle cell disease pathophysiology and clinical variability. This higher frequency of endothelin-1 homozygous mutant variants in African American controls reveal a potential unrelated role, probably hitherto unknown, of this variant in possibly other disease conditions but not sickle cell disease.[25,31] This difference, compared to our African population potentially also reflects the role of ancestry, traceable either to the Afroasiatic and Nilo-Saharan speaking populations of coastal West Africa or the Niger-Kordofanian groups of inland West Africa.

Designing haplotype tables from previous report,[19] four types of eNOS and endothelin-1 gene haplotypes were observed between sickle cell and control groups. All haplotype combinations were represented, at varying rates (Table 1). The highest frequencies, combines the wild-type homozygotes for eNOS −786T>C and eNOS 298Glu>Asp and ET-1 5665G>T homozygote mutant (41.9% versus 57.3% for sickle cell disease and control groups), followed by homozygote wild-type for all three polymorphisms (33.6% versus 16.7% for sickle cell disease and controls respectively). There is a significant difference in haplotype frequencies between case and controls ($p < 0.01$). The significant disparity in endothelin-1 gene polymorphisms between cases and controls in United States but not in Africa displays either a potential benefit on disease pathology among African Americans, or a deleterious effect among Africans, with consequent implications with genetic haplotypes.

This current observation of significant diversity in endothelin-1 polymorphism between African American sickle cell disease and control groups deserves further examination. To do this, increasing the sample size and further characterization alongside specific clinical parameters, disease stratification (severe versus mild) and sickle cell genetic haplotypes would provide a great benefit in elucidating their clinical and therapeutic implications in disease pathophysiology. Our report shows that endothelial nitric oxide synthase variants are less frequent with no significance among patients with sickle cell disease either in Africa[20] or United States, while there is a potential role for endothelin-1 polymorphism in delineating clinical variability or disease pathophysiology, not seen in Africa but in United States.

Author contributions

BNT conceived, designed experiments and drafted the manuscript; KGN and SEA carried out genotyping and restriction digestion; CKN contributed to the discussion and scientific content. All authors read and approved the final version of the manuscript.

Conflicts of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.gendis.2016.09.002.

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