Genetic predictors for stroke in children with sickle cell anemia

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Abstract

Stroke is a devastating complication of sickle cell anemia (SCA), affecting 5-10% of patients before adulthood. Several candidate genetic polymorphisms have been proposed to affect stroke risk but few have been validated, mainly because previous studies were hampered by relatively small sample sizes and absence of additional patient cohorts for validation testing. To verify the accuracy of proposed genetic modifiers influencing stroke risk in SCA, we performed genotyping for 38 published single nucleotide polymorphisms (SNPs), as well as $\alpha$-thalassemia, G6PD A$^{-}$ variant deficiency, and $\beta$-globin haplotype in two cohorts of children with well-defined stroke phenotypes (130 stroke, 103 non-stroke). Five polymorphisms had significant influence ($p<0.05$): SNPs in the ANXA2, TGFBR3 and TEK genes were associated with increased stroke risk, while $\alpha$-thalassemia and a SNP in the ADCY9 gene were linked with decreased stroke risk. Further investigation at these genetic regions may help define mutations that confer stroke risk or protection in children with SCA.
Introduction

Stroke is a catastrophic complication of sickle cell anemia (SCA), occurring in 5-10% of pediatric patients.\textsuperscript{1} This high incidence of cerebral vasculopathy highlights the need for prognostic tests to identify which children with SCA are at greatest risk for developing these irreversible cerebrovascular events. Accurate identification of high-risk children would allow early treatment to prevent the development of cerebrovascular disease and especially to prevent primary stroke events.

Currently, the only clinically useful prognostic tool available for primary stroke prevention is periodic screening of time averaged mean velocities (TAMV) in the distal internal carotid arteries and middle cerebral arteries using Transcranial Doppler (TCD) ultrasonography.\textsuperscript{2} Children with abnormal TCD (defined as TAMV $\geq$200 cm/s) have an approximately 44-fold higher risk of developing primary stroke than those with normal TCD velocities (TAMV $<$170 cm/s).\textsuperscript{3} Children with abnormal TAMV are offered intervention with blood transfusions that lowers risk of stroke by more than 90%.\textsuperscript{4} However, universal TCD screening programs are not available at all pediatric institutions, and the 200 cm/sec threshold is not absolute since children with conditional TCD velocities (TAMV 170 – 199 cm/s) also have an elevated risk of developing stroke.\textsuperscript{3} The limitations of TCD to accurately identify all SCA patients who will develop cerebrovascular complications, as well as reluctance for investigators and families to commit to an indefinite chronic transfusion program, exposes the need for a more sensitive and specific panel of stroke prediction biomarkers.

Previous evidence from sibling-pair analysis documented a genetic contribution to stroke risk in SCA.\textsuperscript{5,6} Several retrospective studies have also demonstrated that specific genetic modifiers may influence the development of cerebrovascular disease in individuals with SCA.\textsuperscript{7-9}
Although previous studies have been hampered by relatively small sample sizes and the absence of validation studies using additional cohorts of patients, they have generated a list of putative genes and polymorphisms that may influence stroke risk (Table 1). To validate the accuracy and prognostic value of these specific genetic modifiers, we genotyped these polymorphisms in two large prospective cohorts of children with SCA and well-phenotyped cerebrovascular disease. We also assessed the effects of G6PD A- variant deficiency, α-thalassemia status, and β-globin gene haplotype on stroke risk. We have found consistent association of five polymorphisms with stroke and further investigation at these genetic regions may help define mutations that confer stroke risk or protection in children with SCA.

Methods

Subjects

Pediatric patients with SCA and documented primary stroke (n=130, average age at stroke 5.8 ± 2.8 years) were recruited through the Stroke With Transfusions Changing to Hydroxyurea (SWiTCH, NCT00122980) study (average age at enrollment 12.9 ± 4.0 yrs). All stroke patients had an independently documented primary stroke event in combination with confirmed MRI evidence showing ischemic stroke. Any patient with MRI evidence of hemorrhagic stroke was excluded. As a control non-stroke group, pediatric SCA patients without prior hydroxyurea treatment (n=103, average age 10.2 ± 3.5 years) enrolled in the Hydroxyurea Study of Long-Term Effects (HUSTLE, NCT00305175) were selected. All participants in the HUSTLE cohort were over 5 years old at enrollment and none had a previous clinical stroke, elevated TCD velocities or any evidence of silent infarcts based on their baseline brain MRI. This study has IRB-approval at St. Jude Children’s Research Hospital (SWiTCH, #CR00000555).
**SNP Genotyping and Sequencing**

Thirty-eight single nucleotide polymorphisms (SNPs) with published associations for stroke risk were genotyped, along with α-thalassemia, G6PD A− variant deficiency and β-globin haplotype of each patient. Genomic DNA TaqMan PCR was performed on an Applied Biosystems StepOne instrument (AB, Foster City, CA). The G6PD variant A and deficient variant A− were determined using the combination of the rs1050829 (N126D) and rs1050828 (V68M) SNPs. Sequencing was performed by Big Dye® Terminator (v3.1) chemistry using capillary electrophoresis on an Applied Biosystem 3730XL DNA Analyzer. The α-thalassemia status of each patient was determined as previously described.

**Statistical analysis**

The minor allele of each polymorphism was evaluated for potential association with stroke via two 2x2 phenotype x genotype contingency tables. Phenotypes were classified as “no stroke” (HUSTLE subjects) or “stroke” (SWiTCH subjects). Separate contingency tables tested for a recessive association (genotype categories {AA, Aa} vs. {aa}) and a dominant association ({AA} vs. {Aa, aa}). One-tail Fisher’s Exact Test (FET) was used when there was a published orientation (increased or decreased stroke risk) for the polymorphism; two-tail FET if orientation was not published. Any SNP with a p-value below 0.05 and associated with the same effect on stroke (increased or decreased risk) as previously published was considered significant, although none of the p-values remained statistically significant when adjusted for the number of multiple comparisons (n=82).
Results and discussion

We genotyped 38 candidate SNPs in 22 genes that have previously been associated with stroke risk (Table 1), along with G6PD A\textsuperscript{-} variant (A\textsuperscript{-}), \(\alpha\)-thalassemia status, and \(\beta\)-globin gene haplotypes. In our large well-phenotyped study (n=233 subjects), we found that only 4 of the 38 SNPs were significantly associated with stroke risk (Table 2), all with the same effect on stroke (increased or decreased risk) as previously published. Specifically, ANXA2 rs11853426, TEK rs489347 and TGFBR3 rs284875 variants were all associated with increased stroke risk (odds ratio > 1). Conversely, ADCY9 rs2238432 was associated with decreased stroke risk (odds ratio < 1). As previously reported\textsuperscript{12}, presence of \(\alpha\)-thalassemia (3.7kb gene deletion) was also associated with decreased stroke risk (p=0.009), with the deletion being nearly twice as frequent in the control group as the stroke cohort (Table 2). In contrast, the G6PD A\textsuperscript{-} variant was not linked with stroke risk (p=0.62; 16.4\% and 12.9\% G6PD A\textsuperscript{-} variant males were in the control and stroke cohorts, respectively), contrary to a recent report that showed elevated TCD velocities and stenosis was associated with G6PD enzyme deficiency\textsuperscript{13}. Classical \(\beta\)-globin gene haplotypes were determined using 5 classical SNPs within the \(\beta\)-globin locus\textsuperscript{14}, resulting in alleles primarily representing the four classical African haplotypes including Benin, Central African Republic, Senegal and Cameroon. None of the \(\beta\)-globin haplotypes were associated with stroke (Table 2).

The objective of this study was to corroborate published genetic modifiers that may aid prediction of stroke in children with SCA. Our results are consistent with the presence of \(\alpha\)-thalassemia being associated with decreased stroke risk. Of other previously reported polymorphisms, the majority of the candidate SNPs were not significantly different between our
cohorts. Only 4 out of 38 SNPs were corroborated in our independent study. These findings highlight the need for careful validation studies before any published genetic modifiers are used to prospectively guide treatment or clinical care.

The validated rs2238432 SNP is located within the ADCY9 gene, a membrane bound adenylate cyclase. The ADCY9 gene has highest expression in the brain and is critical for neuronal signalling. The rs489347 SNP is located in the TEK gene, which encodes a tyrosine kinase expressed on endothelial cells and hematopoietic stem cells. This gene has been shown to help prevent and recover from stroke events. The rs11853426 SNP is located in the annexin A2 gene, which regulates cell surface plasmin generation and may affect the hypercoaguable state of SCA. Finally, the rs284878 SNP resides within the TGFBR3 gene, a transforming growth factor (TGF)-beta receptor. Mutations in TGFBR3 have been linked with cerebrovascular disease. However, it should be emphasized that all of these validated SNPs are intronic and no true mechanistic insights can be derived from our findings; these SNPs may be linked to other nearby genetic polymorphisms that actually modify the stroke risk. Further investigations at these genetic regions may help define specific mutations that actually affect biological functions and confer stroke risk or protection in children with SCA.

While this study examined stroke in a group of patients that may be related to macro- or micro-vasculopathy, thromboses or collapses, we have confirmed that there is a genetic component to development of cerebrovascular disease in SCA. We have validated that 4 SNPs and α-thalassemia are significantly associated with stroke. Our ambition is to combine these gene polymorphisms with larger agnostic genome-wide association studies searches for predictive stroke markers to identify all risk markers for children with SCA. At that time, a genetic “risk profile” could be considered that might complement other risk factors such as TCD screening.
velocities. Ultimately, early and accurate identification of at-risk children with SCA can lead to appropriate use of therapeutic interventions for prevention of stroke.

Acknowledgements

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Author Contributions

J.M. Flanagan analyzed the data and wrote the paper. D.M. Frohlich and T.A. Howard, performed experiments. W.H. Schultz, C. Driscoll, R. Nagasubramanian, N.A. Mortier, A.C. Kimble and B. Aygun helped design the study and collected samples. R.J. Adams and R.W. Helms performed statistical analyses. R.E. Ware designed the study and wrote the manuscript.

Conflict of Interest

The authors have no conflict of interests to declare.
Reference List


Table 1. Published candidate genetic modifiers reported to influence cerebrovascular disease in children and adults. Thirty-eight SNPs in 22 genes have previous association with stroke. The suspected role of the gene in affecting risk of stroke is given in the gene ontology.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Title</th>
<th>SNP</th>
<th>Reference</th>
<th>Gene Ontology</th>
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<td>IL4R</td>
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<td>rs1805015</td>
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Table 2. Polymorphisms associated with the incidence of stroke and validated in the SWiTCH and HUSTLE cohorts. The minor allele and the minor allele frequency are given for each SNP. Significance between the control (HUSTLE) and stroke (SWiTCH) groups was tested using Fisher’s Exact Test (FET) under the dominant or recessive genetic models. All SNPs matched the published orientation of association with stroke risk (increased or decreased risk) and were tested by a one-tail FET. The HbA2 polymorphism is the Δ3.7kb α-thalassemia single gene deletion. Abbreviations for the β-globin haplotypes are as follows: BEN for Benin, SEN for Senegal, CAR for the Central African Republic (Bantu), CAM for Cameroon, and Atypical for any haplotype differing from the classical haplotypes.
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