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Endothelial Nitric Oxide Synthase Tagging Single Nucleotide Polymorphisms and Recovery from Aneurysmal Subarachnoid Hemorrhage

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Abstract

Aneurysmal subarachnoid hemorrhage (SAH) is a hemorrhagic stroke subtype with a poor recovery profile. Cerebral vasospasm (CV), a narrowing of the cerebral vasculature, significantly contributes to the poor recovery profile. Variation in the endothelial nitric oxide (NO) synthase (*eNOS*) gene has been implicated in CV and outcome after SAH. The purpose of this project was to explore the potential association between three *eNOS* tagging single nucleotide polymorphisms (SNPs) and recovery from SAH. We included 195 subjects with a diagnosis of SAH and DNA and 6-month outcome data available but without pre-existing neurologic disease/deficit. Genotyping was performed using an ABI Prism[®] 7000 Sequence Detection System and TaqMan[®] assays. CV was verified by cerebral angiogram independently read by a neurosurgeon on 118 subjects. Modified Rankin Scores (MRS) and Glasgow Outcome Scale (GOS) scores were collected 6 months post hemorrhage. Data were analyzed using descriptive statistics, ANOVA and chi-square analysis as appropriate. The sample was primarily female ($n = 147$; 75.4%) and Caucasian ($n = 178$; 91.3%) with a mean age of 54.6 years. Of the subjects with CV data, 56 (47.5%) developed CV within 14 days of SAH. None of the SNPs individually were associated with CV presence; however, a combination of the three variant SNPs was significantly associated with CV ($p = .017$). Only one SNP (RS1799983, variant allele) was associated with worse 6-month GOS scores ($p < .001$) and MRS ($p < .001$). These data indicate that the *eNOS* gene plays a role in the response to SAH, which may be explained by an influence on CV.

Keywords

Subarachnoid hemorrhage; cerebral vasospasm; nitric oxide; genotype; outcome

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Subarachnoid hemorrhage (SAH) is a devastating hemorrhagic stroke subtype associated with mortality rates of 20–70% and disability affecting up to 20% of survivors (Broderick, Brott, Duldner, Tomsick, & Leach, 1994; Dumont et al., 2003; Hop, Rinkel, Algra, & van Gijn, 1997; Ingall, Asplund, Mahonen, & Bonita, 2000; Kassell, Sasaki, Colohan, & Nazar, 1985; Olafsson, Hauser, & Gudmundsson, 1997). SAH is most commonly due to the rupture of an aneurysm in the large vessels of the circle of Willis that provide blood flow to the cerebral tissue and results in blood in the subarachnoid space around the brain. While the initial increase in intracranial pressure associated with an increase in blood volume in the cranial vault accounts for much of the early mortality, secondary injury occurring in the days to weeks after the hemorrhage contributes significantly to disability and mortality after SAH (Broderick et al., 1994; Dumont et al., 2003; Kassell et al., 1985; Olafsson et al., 1997).

Cerebral ischemia related to cerebral vasospasm (CV) is the most common delayed secondary injury occurring after SAH and significantly contributes to poor outcome. Within days after the initial hemorrhage, the blood or breakdown products of the blood in the cerebrospinal fluid bathing the blood vessels in the subarachnoid space alter vasomotor tone and promote vasoconstriction of the vessels leading to a spasmodic state in up to 70% of patients surviving the initial hemorrhage (Adams, Kassell, Torner, & Haley, 1987; Al-Yamany & Wallace, 1999; Dehdashti, Mermillod, Rufenacht, Reverdin, & de Tribolet, 2004; Dorsch, 2002). CV, a restriction in the internal lumen diameter of the cerebral blood vessels, can occur in one or multiple cerebral blood vessels and often lasts for days (Kassell et al., 1985; Kassell, Torner, Jane, Haley, & Adams, 1990). The decrease in blood flow associated with CV leads to hypoxia and decreased nutrient delivery and frequently to ischemia and infarction, which if not reversed, results in stroke (Haley, Kassell, Alves, Weir, & Hansen, 1995; Haley, Kassell, & Torner, 1992; Lanzino & Kassell, 1999; Ohman, Servo, & Heiskanen, 1991). Clinical symptoms occur in 17–40% of patients experiencing CV (Lee et al., 2006; Mascia et al., 2003; Qureshi et al., 2000), suggesting that there are unknown factors influencing vasomotor tone and neuronal response involved. To date, only two predictors of CV have been identified – younger age (Torbey et al., 2001; Treggiari-Venzi, Suter, & Romand, 2001) and the amount and distribution of blood noted in the intracranial space on computed tomography (CT) scan (Fisher grade and Fisher-Claassen grade) (Claassen et al., 2001; Fisher, Kistler, & Davis, 1980). While these predictors can identify patients at greater risk, they are not sensitive or specific. Other variables that have been implicated in the development of CV include hypertension, cigarette smoking and cocaine use (Conway & Tamargo, 2001; Lasner et al., 1997; Qureshi et al., 2001; Weir et al., 1998).

However, many individuals who are not at risk for CV based on this profile will develop CV. Current management involves attempting to prevent CV with oral Nimodipine (a calcium channel blocker; Allen et al., 1983) and, in some institutions, statins and magnesium infusion. Additional therapy to prevent and decrease the ischemia associated with CV includes hypervolemia, hypertension and hemodilution. However this therapy, termed “Triple H” therapy, is difficult to accomplish as many patients recovering from SAH also have comorbidities that limit its use (e.g., cardiac or pulmonary disease; Sawada et al., 1997; Sayama, Liu, & Couldwell, 2006). There are also problems with other interventions aimed at reducing CV. Direct mechanical or pharmacologic (e.g., Papavarine) opening of the vessels with primary interventions are also problematic in that they are not consistently effective in preventing the negative consequences of CV in all patients (Sawada et al., 1997; Sayama et al., 2006).

Because the physiologic mechanisms driving the development of CV after SAH are unknown, the search for more effective treatment has been difficult. CV most often occurs between 3 and 7 days after SAH (Dorsch, 2002; Gerard, Frontera, & Wright, 2007; Haley, Kassell, Apperson-Hansen, Maile, & Alves, 1997; Haley et al., 1992), suggesting that it is a response to the release of one or more byproducts of blood breakdown in the subarachnoid space, where the exterior

surfaces of cerebral vessels are exposed to blood and products of its breakdown. The breakdown of blood in the subarachnoid space results in the release of oxyhemoglobin-derived free radicals, which inhibit ATP-dependent calcium pumps (Arai, Takeyama, & Tanaka, 1999; Macdonald & Weir, 1991; Macdonald et al., 2001; Macdonald et al., 1991). This development is believed to lead to alteration in blood vessel tone and possibly influences development of CV (Fujii & Fujitsu, 1988). Oxyhemoglobin has a direct effect on the arterial vessel wall, causing vessel spasm, and mediates the release of free radicals, such as NO, and vasoactive substances promoting a spasmodic state (Macdonald & Weir, 1991).

Recent work has implicated nitric oxide (NO) as a mediator of vasomotor tone that may impact CV development after SAH. NO is an endogenous vasodilator formed by its cleavage from L-arginine by nitric oxide synthases (NOS). The formation of NOS, and thereby NO, is driven by gene transcription activated by many intra- and extracellular stimuli. NO availability is decreased after SAH (Jung et al., 2004; Kajita et al., 1994; Kim, Schini, Sundt, & Vanhoutte, 1992). NOS is produced by neurons (nNOS), endothelial cells (eNOS) and other cells (inducible NOS; iNOS). The decreased availability of NO after SAH may be related to a decrease in NOS production and/or activity and/or NO disruption or scavenging (Iuliano, Pluta, Jung, & Oldfield, 2004; Jung et al., 2004; Kim et al., 1992). After SAH, eNOS function is impaired in cerebral vessels (Iuliano et al., 2004), limiting any increase in vessel relaxation induced by the eNOS-associated increase in NO production. Administration of NO donors and NOS metabolites have shown efficacy in decreasing angiographic CV (Afshar, Pluta, Boock, Thompson, & Oldfield, 1995; Hino et al., 1996; Pluta, Oldfield, & Boock, 1997; Thomas, 1997; Thomas & Rosenwasser, 1999; Thomas et al., 1999; Tierney et al., 2001). However, the transformation of these clinical trials into widespread clinical treatment was limited by the short half-life of NO, side effects and potential toxicity (Afshar et al., 1995; Dietrich & Dacey, 2000; Hino et al., 1996).

The production of NOS is genetically driven by three genes. Specifically, endothelial NOS (eNOS) is produced by endothelial cells and this production is driven by the *eNOS* gene on chromosome 18. Neuronal NOS (nNOS) is produced by neurons and this enzyme production is driven by the *nNOS* gene. Inducible NOS is produced by a variety of cell types including endothelial cells and neurons during periods of exposure to hypoxia or other environmental stimuli and production is driven by the *iNOS* gene. The *eNOS* gene is primarily active in the endothelial cells of the vasculature. These cells act to increase NOS production at the vascular level, increasing NO production and ultimately vasodilation. In this way, the endothelial cells influence delivery of blood flow to tissues in need by expressing eNOS. In humans with coronary artery disease, individuals with the variant of the *eNOS* single nucleotide polymorphism (SNP) in the promoter region (T-687C) and in the G894T SNP had decreased endothelium-dependent relaxation (Erbs et al., 2006). Additional studies have found the *eNOS* genotype to be inconsistently implicated in the development of CV. A study by Khurana and associates (2004) on 28 human subjects recovering from SAH found that an SNP in the *eNOS* promoter region (T-786C) was associated with increased risk of CV (Khurana et al., 2004). Song and colleagues found the T-786C was not associated with SAH or CV but was associated with poorer outcomes in a study of 136 subjects with SAH and 110 control subjects (Song et al., 2006). Berra and colleagues showed that eNOS expression was upregulated after SAH and that there was a further increase in expression in subjects with poor clinical conditions (Berra, Carcereri De Prati, Suzuki, & Pasqualin, 2007). No further work has been done to clarify these conflicting findings.

In the study described here, we used tagging SNPs to measure genetic variation in the *eNOS* gene. A tagging SNP (tSNP) is an SNP within a gene that serves as a surrogate for all genetic variation in a region of DNA within the gene. By measuring genetic variation with these surrogates, it is possible to measure the influence of functional SNPs, such as T-786C, as well

as genetic variance that may influence phenotype but has not yet been identified. The three tSNPs that we chose are representative of all known variation within the *eNOS* gene at the time this study was done. The overall purpose of the current project is to explore the potential association between three *eNOS* tSNPs and recovery (CV and functional outcome) from SAH.

Methods

Design

For this study, we used a prospective, between-group, within-subject repeated measures design to identify the potential relationship between NOS genotypes and outcome (CV development and gross functional outcome) in individuals recovering from SAH while controlling for covariates such as age, severity of hemorrhage and comorbidities.

Participants

Participants comprised patients, age 21–75, admitted to the University of Pittsburgh Medical Center (UPMC) neurovascular ICU with a diagnosis of aneurysmal SAH verified by CT scan (Fisher grade ≥ 2) and aneurysm presence and location verified by cerebral angiogram or computed tomographic angiogram and no pre-existing chronic neurologic diseases or deficits. In order to capture patients most at risk for CV, we excluded patients with non-aneurysmal SAH from this study. At our facility lumbar puncture and cerebral spinal fluid (CSF) analyses are used to determine presence of SAH only after a negative CT scan. All patients in this study had a CT scan positive for SAH on admission. A total of 195 patients meeting inclusion criteria had DNA and outcome data available were enrolled in this study.

Collection of Demographic and Medical Condition Data

Project personnel extracted demographic variables including age, gender, and race from medical records. Upon admission to the UPMC, all participants had a head CT scan and the neurosurgeon assigned a Fisher grade. The Fisher grade is a measure of the amount and distribution of blood noted in the intracranial space on CT scan. While the tool has a range of 1–4, a Fisher grade of 3 is most highly correlated with CV development (Fisher et al., 1980). All participants had a cerebral angiogram or computed tomographic angiogram to identify aneurysm size and location and, if appropriate, coil embolization for aneurysm securement. For patients not having coil embolization, surgical clipping for aneurysm securement was performed as soon as possible. The Hunt and Hess score was determined on admission by the neurosurgeon responsible for clinical care, and project personnel extracted this score from medical records. Hunt and Hess score is a tool used to quantify clinical exam on admission and predict long-term functional outcome. Scores range from 1 to 5, with higher scores being predictive of poorer outcome (Hunt, 1983).

Genotyping

We collected a single blood sample from each participant into EDTA-containing vacutainer tubes and processed it for white blood cell removal. DNA was extracted from the first available blood sample using a simple salting out procedure (Miller, Dykes, & Polesky, 1988). To assess all of the genetic variability within the *eNOS* gene as well as the promoter and 3' flanking region, we identified a total of three tSNPs with a minor allele frequency $\geq 20\%$ with an $r^2 \geq .80$ using build 35 of the Hapmap database. The three tSNPs identified were rs1799983, rs1800779, and rs3918188. We used an ABI Prism® 7000 Sequence Detection System to conduct allele discrimination using TaqMan® assays (Applied Biosystems, Foster City, CA). Due to the sample size available and genotype distribution, we dichotomized the sample into groups based on variant presence (homozygote for variant allele combined with heterozygotes) versus variant absence (homozygote for wild type allele) for each SNP analysis.

CV Verification

The presence or absence of CV was verified by cerebral angiography read by a neurosurgeon. The population included in this sample had CV angiography to monitor for CV presence routinely on Day 5 after SAH. CV presence was assessed in the anterior cerebral, anterior communicating, middle cerebral, internal carotid, posterior cerebral, posterior communicating, vertebral and basilar arteries and classified as cerebral vasospasm negative (0–24% constriction in any portion of any of these vessels) or cerebral vasospasm positive ($\geq 25\%$ constriction in any portion of any of these vessels). CV angiograms were coded for percent of vessel constriction for research purposes at a later time. Cerebral angiography is the gold standard for verification of CV.

Functional Outcome

Glasgow Outcome Scale (GOS) and Modified Rankin Score (MRS) were administered by a neuropsychological technician during a face-to-face or telephone interview at 3, 6, 12 and 24 months following SAH. The neuropsychological technician was blinded to the results of NOS genotyping and CV.

The GOS, a clinical observation scale, categorizes functional outcomes into five levels: 1--death, 2--persistent vegetative state, 3--severe disability, 4--moderate disability, 5--good recovery (Jennett, & Bond, 1975).

The MRS is another clinical observation scale, which categorizes functional outcomes into seven levels: 0--no symptoms; 1--no significant disabling symptoms. no significant disability despite symptoms, able to carry out all usual duties and activities; 2--slight disability, unable to carry out all previous activities but able to look after their own affairs without assistance; 3--moderate disability, requiring some help but able to walk without assistance; 4--moderate/severe disability, unable to walk without assistance and unable to attend to own bodily needs without assistance; 5--severe disability, bedridden, incontinent and requiring constant nursing care and attention; and 6--death (Sulter et al., 1999; van Swieten, Koudstaal, Visser, Schouten, & van Gijn, 1988; Wilson et al., 2005).

Data Analysis

Basic descriptive statistics were used to identify differences in demographic characteristics based on genotype. Analysis of variance and Chi square analyses were used to determine differences in outcome variables by genotype while controlling for age and Fisher grade.

Results

Sample

There was a total of 195 subjects with DNA and outcome data included in these analyses. Subjects were primarily Caucasian and female with a mean age of 54.6 years. The Fisher grade mode for the entire sample was 3, indicating that over half of the sample was at the highest risk for development of CV. The Hunt and Hess score mode for the entire sample was also 3, with 81% of the sample having scores of 1–3 indicating low risk of poor outcome. Demographic data and admission severity of hemorrhage scores are presented in Table 1. The full range of Fisher grades and Hunt and Hess scores for the entire sample are presented in Table 2. Genotype frequencies for each of the three SNPs are presented in Table 3. The three SNPs were separately examined for adherence to Hardy-Weinberg equilibrium with a goodness-of-fit test and were found to be in equilibrium for the entire sample.

CV

There were 118 subjects with available CV data. Of these, 56 (47.5%) developed CV within 14 days of SAH. There were no significant differences in demographic variables based on CV presence. Individuals who developed CV more often had a Fisher grade of 3 than those who did not. See Table 1 for demographic and medical condition characteristics by CV presence. Number and percentage of subjects in each Fisher grade category and Hunt and Hess score category by CV presence are presented in Table 2. None of the SNPs examined were individually associated with CV presence; however the combination of three SNPs was associated with CV presence. Subjects with variant copies of rs1799983, rs1800779, and rs3918188 more often developed CV ($p = .017$) than the other subjects.

GOS

We had 6-month GOS data available for all 195 subjects. Most subjects ($n = 70$, 40.4%) had a GOS of 5, indicating good recovery and resumption of normal life despite minor deficits. Only one SNP (rs1799983) was associated with 6-month GOS, with individuals with at least one copy of the variant allele having poorer outcomes ($p < .001$). See Figure 1 for GOS by rs1799983 genotype.

MRS

We had 6-month MRS data for 167 subjects. Of those, 98 (55.1) had MRS scores of 0 or 1, indicating no symptoms or no significant disability despite minor symptoms. Only one SNP (rs1799983) was associated with 6-month MRS, with subjects with at least one copy of the variant allele having poor MRS ($p < .001$). See Figure 2 for MRS by rs1799983 genotype.

Discussion

This study had two important findings: 1) the three tagging SNPs within the *eNOS* gene are not individually associated with the development of CV; the combination of variant alleles of the three SNPs, however, is; 2) the variant allele of the tagging SNP rs1799983 is associated with a poorer outcome as measured by both GOS and MRS. Thus, though the SNPs within the *eNOS* gene do not independently explain CV, when considered together they may serve as a predictor for CV.

Researchers have previously explored *NOS* gene expression (mRNA) after SAH. In a study of human brain tissue taken from 23 subjects recovering from SAH, *eNOS* and *iNOS* expression were increased, while *nNOS* expression was unchanged compared to control samples (Berra et al., 2007). This altered expression of *NOS* genes may be driven by genetic variability. In particular, variation in either the coding regions or the promoter regions may alter mRNA levels, protein levels and functional recovery of patients after SAH. This study explored all variation within the *eNOS* gene and, as such, better reflects the genetic variance that may be influencing mRNA, eNOS production and amount of eNOS produced. Our findings suggest that there is variance in other regions of the *eNOS* gene that may be influencing vasomotor tone and recovery from SAH via an as yet unidentified mechanism not associated with the functional SNP (T-786C).

There have also been previous studies exploring the relationship between CV and *eNOS* genotypes. Previous work has focused primarily on a single known functional SNP in the *eNOS* gene (Khurana et al., 2004). The findings of the current study support those of the studies by Khurana and associates (2004) and Song (Song et al., 2006), who found that *eNOS* genotype was associated with CV after SAH. While our work did not find an association between the region containing the SNP explored by this work and CV, the combination of SNPs was predictive of CV development. Utilizing tagging SNPs allowed us to assess variation

throughout the entire gene sequence and the relationship with CV, adding strength to our analyses.

Research in other areas has implicated variation in the T-786C SNP in hypertension (Kuhlencordt et al., 2001; Wang & Wang, 2000), myocardial infarction (Hingorani, 2001; Wang et al., 1996), and coronary vasospasm (Nakayama et al., 1999), all disorders influenced by vasomotor reactivity. The T-786->C polymorphism results in a 52% reduction in promoter activity (Nakayama et al., 1999). The significant reduction in promoter activity ultimately leads to decreased eNOS protein expression. eNOS production generates active NO, which leads to vasodilation. The 52% reduction in eNOS production resulting from this variant allele, which resides within the haploblock tagged by SNP rs1799983 measured for this study, results in decreased vasodilation (Song et al., 2003). The variant (C) allele at the -786 position is translated such that there is less eNOS produced, which results in a decrease in NO production and decreased vasodilation. In this way, individuals with a variant (C) allele at the -786 position are at increased risk of decreased vasodilation and therefore CV after SAH.

While our study did not find direct association between the rs1799983 variant allele containing this SNP and CV, this tagging SNP was associated with poorer outcome. The -786 SNP may still be influencing CV development or response to CV and ultimately drive the association between the tagging SNP (rs1799983) and poor outcome. Variation in the gene sequence may lead to worse outcomes via alteration of CV risk or response to CV.

The confusion within the literature regarding the role of the T-786C allele and CV may also be influenced by the method of CV measurement. Khurana and associates (2004) defined CV as decline in neurologic status coupled with an elevation in cerebral blood flow velocity (measured by transcranial Doppler ultrasonography). This method of vasospasm measurement underestimates angiographic vasospasm as a diagnosis of CV positive is dependent upon a change in the neurologic exam that results from tissue hypoxia/ischemia. It is, however, a clinically relevant definition as angiographic vasospasm without clinical signs and symptoms is often undiagnosed and untreated. Variation in neuronal response could be mediated by eNOS expression, and this may be the phenomenon found by this body of literature. Ko and colleagues (2008) used only cerebral angiography to define CV and found similar results, showing that decreased *eNOS* expression is directly related to cerebral vessel reactivity. The regional decrease in NO after SAH and the variation in eNOS expression directed by the *eNOS* gene are highly suggestive of the influence of *eNOS* in CV.

It is also possible that the additional genetic variability explored in the current project masked effects. Using tagging SNPs for this analysis was more informative, as we were able to explore more genetic variability than if we had, as with previous studies, examined a single SNP. However there may be additional SNPs within the tagging region with influence on expression or functionality of *eNOS* that cancelled out the relationship at the single SNP level. As previous research has only explored the influence of a single SNP on CV development, it is also possible that variation in other regions of the *eNOS* gene were not adequately explored in previous work but are influential on vasomotor tone and CV development.

A larger sample size may identify relationships more clearly as genetic variability is more accurately represented with more subjects with presence of the minor allele available for analyses. The inconsistency in findings may also be influenced by inheritance. The Ko and Song studies utilized a Korean sample, with anticipated T-786C polymorphism presence lower than in a primarily Caucasian sample, while the Khurana study utilized an American sample (primarily Caucasian). Our sample is comparable to the subjects in the Khurana study, but there may be additional genetic variability exerted by other SNPs with varying frequencies in different populations.

It may be that there is additional influence on NOS and NO production exerted by regions within the *eNOS* gene that are not yet recognized as having functional differences but explain the discrepancy in the literature. As only a combination of SNPs was found to be associated with CV development, additional influence may be exerted by variance represented by tagging SNPs in these regions. Tagging SNPs represent variation within a large sequence of the gene, and the three SNPs in this project describe all variation in the *eNOS* gene. Variance within the regions represented by the tagging SNPs likely leads to variation in either eNOS expression or eNOS function, which influences recovery after SAH.

The variant of the rs1799983 SNP was associated with poorer outcome in both measures (GOS and MRS) used in our study. The functional SNP found to be associated with recovery after SAH by Song et al. (2006) is located within the region represented by rs1799983 SNP and the same region that has previously been associated with CV in the literature. Song and associates found an association between the T-786C SNP and outcome as measured by GOS but not between the SNP and CV, similar to our findings. While there is inconsistency in the literature regarding the relationship between this particular SNP and CV development, the relationship between *eNOS* genotypes and outcome from SAH has not been well described. CV is a cause of secondary injury that is negatively correlated with long-term outcome from SAH; however there are many other events that occur in the recovery process, and *eNOS* is likely influential on long-term recovery in addition to its influence on CV. Certainly alterations in cerebral blood flow may persist beyond the 2-week period during which CV risk is highest. Variation in vasomotor reactivity later in the recovery process, for instance, may influence neuronal viability and long-term recovery. Genetically driven variability in eNOS production may impact cerebral blood flow and neuronal recovery throughout the recovery process. While the altered eNOS expression in individuals developing CV has been documented, there is no literature describing long term eNOS expression dysfunction. Slight decreases in cerebral blood flow due to altered eNOS expression may persist long after the hemorrhage and impair neuronal function leading to poorer outcome. There may be additional, unknown influences on neuronal response to injury and recovery processes that are mediated by eNOS, NOS and NO that have not yet been identified or described.

It does appear, based on our findings in the context of the existing literature, that the decreased NO formation from decreased eNOS production based on genetic variance leads to an environment that is favorable to CV development and poorer outcome after SAH. The variant in the region of the *eNOS* gene that has consistently been implicated in CV development and poorer outcome leads to decreased eNOS production and ultimately a decrease in available NO, which promotes vasoconstriction (Nakayama et al., 1999). This consistent relationship is suggestive of the strong role expression of this gene plays in the recovery process after SAH and could likely lead to therapeutic interventions to decrease CV and improve outcomes.

There are two other genes that influence NOS and NO production. Inducible NOS (iNOS) is expressed by cells of varying types, including neurons, in response to hypoxia and other environmental stimuli. Neuronal NOS (nNOS) is also expressed by neurons in response to hypoxia. The function of nNOS producing neurons at the neurovascular junction is impaired after SAH, and variations in the *nNOS* gene may also exert influence on recovery from SAH (Pluta & Oldfield, 2007). The availability of NO after SAH is likely influenced to a variable extent by each of these genes. Additionally, the role of NOS and NO in development of CV after SAH may be mediated by other mechanisms. NO scavenging may be the source of decreased NO (Pluta, 2006; Pluta & Oldfield, 2007). Alternatively, an increased expression of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor that has been implicated in the development of CV (Jung et al., 2004), might be related to the decrease in available NO. Neither NO scavenging or ADMA expression were measured in this study. Further work exploring the expression of each gene and genotypic expression of these genes will provide

insight into mechanism of CV after SAH and ultimately lead to improved therapeutic strategies to decrease incidence, severity and negative effects of CV.

The limitations of this study include sample size, method of defining CV, and outcome measurements. While our sample size of 195 subjects is one of the largest available (118 for analyses including CV as a variable), it is still a relatively small sample size for a genetic study. The fact that we found significance despite this limitation supports the potential role of these genes' influence on the recovery processes after SAH. We chose angiographic CV rather than symptomatic CV as our study definition because it is the most appropriate definition of CV in relation to the scientific rationale supporting the study. Studies exploring this relationship with symptomatic CV may yield different results and are necessary for clinical relevance. We operationalized our outcome variable as GOS and MRS. These tools measure gross functional outcome but are not specific enough to describe the many neuropsychological impairments that this population may encounter. Further work exploring the role of *eNOS* genotypes on these other outcomes is needed to fully describe this relationship.

Conclusion

These data indicate that the *eNOS* gene plays a role in the response to SAH, which may be explained by an influence on the development of CV. The identification of genetic biomarkers of secondary injury after SAH will likely lead to many changes in the nursing care of patients recovering from SAH. Knowledge about genotypic differences influencing CV will provide insight into mechanism of CV and promote development of therapeutics to improve outcomes. This information will also permit more focused utilization of resources, allowing patients at lower risk to be discharged from an intensive care setting at an earlier time. Knowledge of genotypic differences in long-term outcome will also be instrumental in changing the outcome profile of patients with SAH. In addition to providing insight into other potential mechanisms of recovery from SAH, this knowledge may also lead to the development of genotype-specific therapeutics for use in select populations. Further research validating this work and determining sensitivity and specificity of genotypes in predicting CV and long-term outcome in SAH is needed in order to translate these findings into biomarkers for use in clinical care.

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Figure 1.

Figure 2.

Table 1

Demographic and medical condition characteristics of the entire group and by presence/absence of cerebral vasospasm (CV).

	Total Sample N = 195	CV Present n = 56	CV Absent n = 62	p
Age--mean (SD)	54.6 (10.8)	53.9 (10.4)	55.5 (10.2)	0.40
Caucasian--n (%)	178 (91.3)	50 (89.3)	58 (93.5)	0.41
Female--n (%)	147 (75.4)	46 (82.1)	45 (72.6)	0.21
Fisher grade-- mode (%)	3 (53.8)	3 (58.9)	3 (51.6)	
Hunt and Hess score--mode (%)	3 (34.9)	3 (39.3)	2 (40.3)	

Note. CV data were available for 118 of the 195 subjects enrolled in the study.

Table 2

Hunt and Hess scores and Fisher grades of the subsample with CV data present ($N = 118$) and based on the presence ($n = 56$) or absence ($n = 62$) of cerebral vasospasm (CV).

	Total Sample <i>n</i> (%)	CV Present <i>n</i> (%)	CV Absent <i>n</i> (%)
Hunt and Hess score			
1	16 (13.6)	6 (10.7)	10 (16.1)
2	38 (32.2)	13 (23.2)	25 (40.3)
3	38 (32.2)	22 (39.3)	16 (25.8)
4	19 (16.1)	11 (19.6)	8 (12.9)
5	7 (5.9)	4 (7.1)	3 (4.8)
Fisher grade			
1	0	0	0
2	27 (22.9)	9 (16.1)	18 (29.0)
3	65 (55.1)	33 (58.9)	32 (51.6)
4	26 (22.0)	14 (25.0)	12 (19.4)

There were no significant differences in these variables between the two groups.

Table 3

Genotype frequencies with number of subjects and percentages of the entire sample ($N = 195$) used in this study.

	SNP1 (rs1799983) <i>n</i> (%)	SNP2 (rs1800779) <i>n</i> (%)	SNP3 (rs3918188) <i>n</i> (%)
Wild type homozygous	86 (45.7)	71 (37.8)	24 (12.6)
Heterozygous	84 (44.7)	90 (47.9)	86 (45)
Variant homozygous	18 (9.6)	27 (14.4)	81 (42.4) ^a
Missing	7	7	4

^aThere were more subjects in our sample with the variant allele of SNP3 than there were in the hapmap population used to identify the tagging SNPs.