Review Article

Genetic determinants of cerebral vasospasm, delayed cerebral ischemia, and outcome after aneurysmal subarachnoid hemorrhage

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Despite extensive effort to elucidate the cellular and molecular bases for delayed cerebral injury after aneurysmal subarachnoid hemorrhage (aSAH), the pathophysiology of these events remains poorly understood. Recently, much work has focused on evaluating the genetic underpinnings of various diseases in an effort to delineate the contribution of specific molecular pathways as well as to uncover novel mechanisms. The majority of subarachnoid hemorrhage genetic research has focused on gene expression and linkage studies of these markers as they relate to the development of intracranial aneurysms and their subsequent rupture. Far less work has centered on the genetic determinants of cerebral vasospasm, the predisposition to delayed cerebral injury, and the determinants of ensuing functional outcome after aSAH. The suspected genes are diverse and encompass multiple functional systems including fibrinolysis, inflammation, vascular reactivity, and neuronal repair. To this end, we present a systematic review of 21 studies suggesting a genetic basis for clinical outcome after aSAH, with a special emphasis on the pathogenesis of cerebral vasospasm and delayed cerebral ischemia. In addition, we highlight potential pitfalls in the interpretation of genetic association studies, and call for uniformity of design of larger multicenter studies in the future.

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Introduction

Pathogenesis of Delayed Cerebral Ischemia and Cerebral Vasospasm after aSAH

Delayed cerebral ischemia (DCI) has been implicated as major, potentially preventable contributor to poor outcome after aneurysmal subarachnoid hemorrhage (aSAH) (Hijdra *et al*, 1987). It is a reversible failure of cerebral perfusion that can progress to infarction and has been shown to increase the risk of mortality and severe disability in patients with aSAH (Hijdra *et al*, 1988). Though DCI can result from cerebral vasospasm, not all patients with DCI have reported radiographic vasospasm, suggesting a more complex

set of contributors (Dankbaar *et al*, 2009; Naidech *et al*, 2006; Stein *et al*, 2006a).

Pathogenetic mechanisms leading to DCI are multifactorial and are thought to include cortical spreading ischemia (Dorsch, 1995; Dreier *et al*, 1998, 2006b) and microthrombosis (Stein *et al*, 2006b; Vergouwen *et al*, 2008) in addition to delayed injury resulting from angiographic vasospasm (Dehdashti *et al*, 2004). An observed association between cortical spreading depression and DCI has led to the hypothesis that SAH-induced cortical spreading induces ischemia through microvascular spasm. Animal studies show that erythrocyte breakdown products such as potassium and hemoglobin alter the subarachnoid microenvironment and induce cortical spreading depression with an acute ischemic cerebral blood flow response (Dreier *et al*, 1998). Supportive human evidence comes from studies in which repeated spreading depolarizations with prolonged depression periods, as recorded by subdural electrodes in aSAH patients, proved to be an early indicator

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of DCI, and were associated with hypoperfusion (Dreier *et al*, 2006a, 2009). Another hypothesis implicates microthrombosis in the pathogenesis of DCI (Vergouwen *et al*, 2008). Multiple autopsy studies have identified the presence of microthrombi in the vessels of aSAH patients with DCI (Neil-Dwyer *et al*, 1994; Stein *et al*, 2006a; Suzuki *et al*, 1983). Since the early identification of microthrombosis in DCI, investigators have described a number of mechanisms that promote microthrombosis in SAH, including activation of the coagulation cascade, the inflammatory cascade (Dreier *et al*, 2009; Hirashima *et al*, 1997; Ohkuma *et al*, 1991), the inflammatory response (Levi *et al*, 2004), and fibrinolytic impairment (Ikeda *et al*, 1997).

Vasospasm after aSAH is a complex entity composed of reversible vasoconstriction, coupled with dysfunction of normal cerebral vascular autoregulation (Khurana and Besser, 1997), leading to a reduction of cerebral blood flow and the development of regional cerebral ischemia (Keyrouz and Diringer, 2007). Proximal vessels at the base of the brain are preferentially affected by cerebral vasospasm, however distal arteries may also develop impaired autoregulation and lead to further alterations of blood flow (Soehle *et al*, 2004). The mechanisms underlying the development of cerebral vasospasm after aSAH have been the subject of extensive study, both in animal models and in human observational cohorts (Fisher *et al*, 1980; Horowitz *et al*, 1996; Zabramski *et al*, 1986). The process begins with the presence of breakdown products of blood in the subarachnoid cisterns, which incite a reactive process that ultimately results in spasm of the cerebral vasculature. On a molecular level, initiation of cerebral vasospasm by the putative spasmogens is manifested by both Ca^{2+} -dependent and -independent processes, resulting in abnormal and prolonged constriction of vascular smooth muscle (Laher and Zhang, 2001; Tani, 2002; Tani and Matsumoto, 2004). A considerable body of experimental evidence implicates oxyhemoglobin as the primary spasmogenic agent in the development of vasospasm (Macdonald and Weir, 1991; Nishizawa and Laher, 2005). This is supported by observations of high concentrations of oxyhemoglobin in the cerebrospinal fluid of patients during the vasospasm period after aSAH, and that changes in oxyhemoglobin concentration show vasospasm evolution (Nishizawa and Laher, 2005). Furthermore, erythrocytes have been identified as a necessary component of blood for the induction of experimental cerebral vasospasm (Macdonald and Weir, 1991). However, evidence suggests that vasospasm after aSAH is dependent on multiple factors, as hemoglobin does not produce as severe spasm as whole blood, and hemolysates of erythrocytes are more potent spasmogens than Hb alone (Macdonald, 2001). In addition, arachidonic acid metabolites, endothelins, free radicals, serotonin, adenosine, and bilirubin oxidation products have all been implicated as having causa-

tive functions in the development of vasospasm (Asano *et al*, 1984; Cook and Vollrath, 1995; Sano *et al*, 1980; Suzuki *et al*, 1999).

Inflammation has also been implicated in the pathogenesis of cerebral vasospasm, as well as in the mechanisms of ensuing ischemic injury. For example, rapid upregulation of inflammation-related genes has been observed in spastic arteries (Dumont *et al*, 2003). Elevation of adhesion molecule levels and complement cascade proteins have also been noted in the cerebrospinal fluid and serum of patients after aSAH (Dumont *et al*, 2003; Fassbender *et al*, 2001; Mack *et al*, 2007). Furthermore, monoclonal antibodies directed against intercellular adhesion molecule 1 and the common β -chain of the integrin superfamily reduces vasospasm in a rabbit model (Bavbek *et al*, 1998). Further support for an inflammatory basis underlying SAH pathophysiology comes from several other reports describing an association between cytokines in the cerebrospinal fluid of patients with SAH and increased transcranial Doppler levels, and the attenuation of experimental vasospasm after complement inhibition (German *et al*, 1996).

Delayed Cerebral Ischemia and Vasospasm as Clinical Outcomes after aSAH

The understanding of these multiple pathogenic processes and recent clinical evidence highlight that a critical distinction must be made between end points such as radiographic vasospasm, symptomatic vasospasm, cerebral infarction, DCI, and other functional outcome measures. A number of studies have shown that not all patients with DCI have angiographic vasospasm, and that DCI is associated with poor outcome whereas angiographic vasospasm alone is not (Frontera *et al*, 2009; Schmidt *et al*, 2008). Furthermore, recent results from the CONSCIOUS-1 trial, a phase IIb, randomized, placebo-controlled trial of clazosentan administration (Macdonald *et al*, 2008), provide further evidence of a disconnect between angiographic vasospasm and poor outcome. Clazosentan, a nonpeptide endothelin receptor-A antagonist, was shown in high dose to be associated with a 65% relative risk reduction of moderate and severe vasospasm (Macdonald *et al*, 2008). Despite such a significant reduction in vasospasm, clazosentan-treated patients had no significant reduction in morbidity and mortality within 6 weeks of hemorrhage; secondary end points included cerebral infarct, delayed ischemic neurologic deficit due to vasospasm, and rescue therapy for radiographic vasospasm (Macdonald *et al*, 2008). Additional evidence regarding a disconnect between vasospasm and clinical outcome derives from study of the calcium channel blocker, nimodipine. This agent is the only approved drug that reduces the incidence of poor outcome after aSAH, and a systematic review shows that nimodipine reduces the risk of poor outcome without showing any clear

effect on vasospasm (Feigin *et al*, 1998). Subsequent work suggested that nimodipine increases fibrinolytic activity, lending support for additional mechanisms of ischemic injury other than vascular contraction in patients with aSAH (Roos *et al*, 2001).

The disjunction between the rates of angiographic and clinical vasospasm after aSAH, as well as failure of Clazosentan to improve outcome despite effectively ameliorating vasospasm, has prompted efforts to better elucidate alternative contributors to DCI after aSAH (Hansen-Schwartz *et al*, 2007; Macdonald *et al*, 2008). In theory, genotypic variability may serve to define in part an individual's susceptibility to both the development of vasospasm and the injury incurred secondary to ischemia throughout both the early and chronic course of this disease. Understanding the genetic determinants of vasospasm and DCI may become invaluable in defining the pathophysiology of vasospasm, and may well aid in developing targeted therapies.

Genetic Association Studies of aSAH

Association studies constitute a common approach to investigating the genetic basis of aSAH. In this type of analysis, the frequency of a specific gene allele is determined and compared between aSAH patients and controls. Using this approach, it was found that a large number of genes have been implicated in the development and subsequent rupture of intracranial aneurysm (IAs), many related to structural proteins of the extracellular matrix that are suspected to be deficient in a majority of aneurysms (Nahed *et al*, 2007). These findings are supported by the fact that these matrix proteins are altered in various genetic diseases associated with high rates of aneurysm formation, such as adult polycystic kidney disease (Chapman *et al*, 1992), Marfan syndrome (ter Berg *et al*, 1986), and Ehlers-Danlos syndrome Type IV (de Paepe *et al*, 1988). Although the majority of genetic research has focused on gene expression and linkage studies of these markers as they relate to the development of IA and their subsequent rupture, far less work has centered on the genetic determinants of cerebral vasospasm, the predisposition to DCI, and the determinants of ensuing functional outcome after aSAH. The candidate gene suspects are diverse, and encompass multiple functional systems including fibrinolysis, inflammation, vascular reactivity, and neuronal repair.

The literature implicating a genetic basis for vasospasm, DCI, and other clinical outcomes has to date provided a preliminary framework in the form of small- to medium-sized genetic association studies. Authors have begun to define relevant genetic polymorphisms and their value in predicting clinical end points. However, the existing literature shows significant variability in the both the above-mentioned end points and their definitions, which

highlights at once the wide influence of genetic variability in aSAH and the need for uniformity to define the genetic determinants of outcome more conclusively. To this end, we present a review of the current evidence implicating a genetic basis for outcome after aSAH and discuss potential pitfalls in the interpretation of often conflicting data resulting from genetic association studies.

Materials and methods

All combinations of the following keywords were used to query the PubMed database for potential studies: subarachnoid hemorrhage, outcome, genetics, vasospasm, polymorphism, aneurysm, delayed cerebral ischemia, and delayed ischemic neurological deficit (DIND). The searches were restricted to the time period January 1980 to November 2009. Studies of all design types were included in the initial survey. Reference lists of selected studies and tables of contents from selected journal websites were hand-searched for additional relevant publications. From the 5,310 articles initially retrieved, 640 were selected for more detailed analysis. Studies were only retained if they analyzed one or more genetic polymorphisms in a patient population after aSAH, with vasospasm, DCI, and/or a clinical outcome as outcome measures. Of 640 studies analyzed, 21 original studies met criteria and were included in the present review.

Genetic Outcome Studies of aSAH

Endothelial nitric oxide synthase: Much of the work specifically investigating the genetic underpinning of vasospasm, both angiographic and clinical, has focused on the gene encoding the endothelial isoform of nitric oxide synthase (eNOS) (Pluta, 2005; Table 1). Nitric oxide (NO) normally inhibits inflammation and smooth muscle proliferation, both of which are pathologic changes that occur during cerebral vasospasm. Impairment of NO signaling is evident after SAH in experimental animals (Khurana and Besser, 1997; Weir and MacDonald, 1993), and abnormal cerebrospinal fluid NO levels have been reported in human patients after SAH (Sadamitsu *et al*, 2001). In gene-transfer experiments, eNOS overexpression in animal and human intracranial arteries is vasoprotective after aSAH (Khurana *et al*, 2000, 2002). Multiple investigations have established the functional relevance of human eNOS gene polymorphisms by showing associations of these with increased susceptibility to various pathologies, including coronary vasospasm and the formation of IAs (Khurana *et al*, 2004a; Yoshimura *et al*, 2000b).

Of the known eNOS gene polymorphisms, which include a variable number tandem repeat in intron 4 (Wang *et al*, 1996), a promoter region single nucleotide polymorphism (SNP, T-786C) (Yoshimura *et al*, 2000a), and a coding SNP in exon 7 (G894T) (Hingorani *et al*, 1999), the eNOS T-786C SNP has been most extensively investigated for associations with outcome after aSAH. The initial study used gene microarray technology and samples from 141 subjects; 90 controls were randomly selected from a

Table 1 Genetics studies of vasospasm and delayed cerebral ischemia after aSAH

Endothelial nitric oxide synthase (eNOS)	Starke <i>et al</i> (2008) ^a	Patients with the T-786 SNP of the <i>eNOS</i> gene are more likely to have severe symptomatic or angiographic vasospasm after aSAH
	Ko <i>et al</i> (2008) ^a	T-786C SNP was associated with increased odds of angiographic vasospasm
	Song <i>et al</i> (2006)	No significant association of the eNOS T-786C SNP genotypes with the sizes of ruptured aneurysms or the occurrence of symptomatic vasospasm after aSAH
	Khurana <i>et al</i> (2006) ^a	The eNOS T-786C SNP could be used to significantly differentiate between the presence and severity of symptomatic and asymptomatic radiographic cerebral vasospasm after aSAH
	Khurana <i>et al</i> (2004a, b) ^a	The <i>eNOS</i> gene promoter T-786C SNP predicts susceptibility to post-SAH vasospasm
Plasminogen activator inhibitor-1 (PAI-1)	Ladenvall <i>et al</i> (2009)	The 4G allele of the 4G/5G promoter polymorphism is not associated with outcome (1-year GOS score) after aSAH
	Vergouwen <i>et al</i> (2004) ^a	The 4G allele of the 4G/5G promoter polymorphism is associated with more frequent secondary ischemia after aSAH
Tissue plasminogen activator (tPA)	Ladenvall <i>et al</i> (2009)	The 7351 C>T SNP of the <i>tPA</i> gene is not associated with differences in favorable outcome (1-year GOS score) after aSAH
Thrombin activatable fibrinolysis inhibitor (TAFI)	Ladenvall <i>et al</i> (2009)	The Ala147Thr SNP of the <i>TAFI</i> gene is not associated with differences in favorable outcome (1-year GOS score) after aSAH
Factors V and XIII (FV and FXIII), methylenetetrahydrofolate reductase (MTHFR), prothrombin	Ruigrok <i>et al</i> (2009)	FV Leiden, prothrombin G20210A, MTHFR C677T, FXIII A Val34Leu, FXIII A Tyr204Phe, FXIII B His95Arg genotypes are not associated with differences in delayed secondary ischemia

aSAH, aneurysmal SAH; GOS, Glasgow Outcome Score; SAH, subarachnoid hemorrhage; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats; WT, wild type.

^aStatistically significant differences in outcome of the study.

nationwide adult registry and 51 persons were admitted with aSAH (Khurana *et al*, 2004b). Using multiple logistic regression analysis and adjustments for age, gender, and smoking history, the authors found a significant difference in the frequency of the eNOS variable number tandem repeat polymorphism between patients with aSAH compared with controls ($P=0.002$). Among patients presenting with a Fisher grade 3 aSAH, the eNOS T-786C SNP was also significantly associated with both the presence and the severity of subsequent symptomatic and asymptomatic radiographic vasospasm ($P=0.04$). In addition, in the entire cohort, the presence of at least one C allele exhibited a trend toward association with the development and severity of vasospasm, with an odds ratio (OR) for spasm of 7.1 (95% CI 0.88 to 57.5; $P=0.065$). Although these results appear compelling, it must be noted that the patients and controls differed in terms of the incidence of hypertension, age, gender, and smoking status. Also, only 51 aSAH patients were included in the analysis, which is a relatively small number, and thus these results need independent confirmation. Subsequent studies, however, have provided conflicting results. Song *et al* (2006) analyzed 136 consecutive patients with aSAH as well as 113 controls for the T-786C polymorphism. They found no significant difference between patients and controls about the distribution of the two eNOS T-786C alleles. Also, the authors found no association between genotype and the development of symptomatic vasospasm in these patients.

Multiple logistic regression analysis controlling for age and sex, however, did show that heterozygosity for the C allele was independently associated with unfavorable outcome. The study population in this report consisted entirely of Koreans with no patients homozygous for the C allele. This emphasizes the importance of recognizing that heterogeneity may exist in polymorphism frequency between different ethnic groups and that the findings of genetic studies should be confirmed in multiple study populations.

In another well-designed study, Ko *et al* (2008) prospectively enrolled a large cohort of patients admitted with aSAH ($n=347$) and determined vasospasm end points relative to the presence of each of the three eNOS polymorphisms. Only the T-786C polymorphism was significantly associated with angiographic cerebral vasospasm, with the homozygous CC genotype resulting in an almost tripling of the odds compared with the wild-type genotype (OR=2.97, 95% CI 1.32 to 6.67, $P=0.008$) after adjustment for age, sex, race/ethnicity, Hunt–Hess and Fisher grades. However, no association between eNOS polymorphisms and cerebral infarction was noted, leading the investigators to hypothesize that eNOS polymorphisms contribute to individual susceptibility to angiographic vasospasm.

The latest investigation into associations between eNOS and outcome after aSAH was performed by Starke *et al* (2008). In this study, 77 patients with aSAH were genotyped,

prospectively enrolled and followed up for the occurrence of symptomatic or angiographic cerebral vasospasm. In a multivariate logistic regression, T-786C genotype was the only factor predictive of vasospasm, with an OR for symptomatic vasospasm of 3.3 in patients possessing one T allele (95% CI 1.1 to 10.0, $P=0.034$), whereas for homozygous TT the OR was higher (10.9). When angiographic vasospasm was considered as the outcome variable, patients developing vasospasm were 3.6 times as likely to possess a single T allele (95% CI 1.3 to 9.6, $P=0.013$) and 12.6 times as likely to be homozygous TT. Furthermore, patients with severe spasm requiring endovascular therapy were also more likely to be heterozygous or homozygous for the T allele (for heterozygous: OR 3.5, 95% CI 1.3 to 9.5, $P=0.016$; for homozygous: OR 12.0). Despite a strong association between possessing a T allele and the development of vasospasm, there was no association between genotype and infarction secondary to vasospasm in this analysis.

These interstudy differences also highlight the lack of understanding of the contributions of the various eNOS haplotype combinations to the pathophysiology of vasospasm after aSAH. One attempt to explain this discrepancy is that endothelial cell damage as a result of aSAH leads to a decrease in expression of the eNOS gene, ultimately leading to vasospasm, whereas a compensatory vasodilatory mechanism involving increased cerebral production of NO by iNOS occurs downstream (Pluta, 2005). The implication of NO in both of these processes may explain why it has been difficult to determine the exact role of NO in aSAH (Hino *et al*, 1996). Also, the preponderance of the evidence in support of a role for eNOS in vasospasm uses angiographic spasm as the end point of interest, highlighting again the disjunction of vasospasm with clinical outcome described previously. Regardless, the genetic association studies implicating eNOS polymorphisms in mediating vasospasm after aSAH should provide stimulus for further work to delineate the role of NO in this disease, and to define the relationship between eNOS SNPs and expression of nitric oxide synthetase and NO in human patients.

Fibrinolysis-related genes: In keeping with the integral involvement of microvascular thrombosis in the pathogenesis of cerebral ischemia (Siesjo, 1992), and the involvement of microthrombi in DCI after aSAH (Suzuki *et al*, 1990; Vergouwen *et al*, 2008) several investigators have attempted to determine associations between fibrinolytic genes and outcome (Table 1). In one study, Vergouwen *et al* (2004) investigated whether the plasminogen activator inhibitor-1 (PAI-1) 4G allele of the 4G/5G promoter polymorphism influences outcome after 126 patients with aSAH. They found that secondary ischemia occurred more often in patients harboring a 4G allele than in patients homozygous for the 5G allele (RR 3.3, 95% CI 1.1 to 10.0). Despite this, they noted no increased rate of rebleeding between the cohorts, but patients with the 4G genotype did tend to have higher risk of poor outcome (determined by 3-month Glasgow Outcome Scale (GOS) scores) relative to the 5G/5G genotype (RR 1.2, 95% CI 0.7 to 2.2). The authors concluded that the 4G allele of the *PAI-1* gene

increases the risk for secondary ischemia after aSAH, likely because of a tendency toward formation of microthrombi in the setting of ischemic stress, and proposed that enhancing fibrinolysis may effectively treat secondary ischemia.

Another recent study investigated associations between several different genes involved in fibrinolysis and functional outcome after aSAH (Ladenvall *et al*, 2009). In this study, 183 patients presenting with aSAH were consecutively recruited and compared with healthy, age, sex, and region-matched controls. Single nucleotide polymorphisms of the tissue plasminogen activator, PAI-1, thrombin-activatable fibrinolysis inhibitor, and factor XIII (*FXIII*) genes were investigated. Despite showing that patients carrying the *FXIII* Leu-34 allele had an increased risk for aSAH itself, no specific SNP or haplotype was associated with outcome after aSAH. Of note, outcome in this study was determined using a particular structured interview developed by Wilson *et al* (1998), with extended GOS (GOS-E) scores dichotomized into favorable (5 to 8) and unfavorable (1 to 4) outcomes, and assessed at one year after aSAH.

Similarly, a study by Ruigrok *et al* (2009) investigated polymorphisms in the Factor V (*FV*), *FXIII*, methylenetetrahydrofolate reductase (*MTHFR*), and prothrombin genes. Although they did find an association between the *FXIII* B His95Arg genotype and an increase in the rate of incidence of aSAH, they did not find any associations with secondary ischemia or rebleeding in that genotype or the *FV* Leiden, Prothrombin G20210A, *MTHFR* C677T, *FXIII* A Val34Leu, and *FXIII* A Tyr204Phe polymorphisms.

Inflammatory genes: As discussed previously, inflammation likely has a pivotal role in both the pathogenesis of cerebral vasospasm and as a downstream mediator of DCI. As such, polymorphisms of inflammatory genes are logical choices for investigation in genetic association studies (Table 2). Proinflammatory cytokines, however, have been shown to provide both detrimental and beneficial effects after brain injury depending on the spatial and temporal aspects of their expression (Stoll *et al*, 2002). Furthermore, proinflammatory cytokines result in increased expression of growth factors, particularly in the subacute and chronic phases of cerebral injury, and thus the role of inflammation after aSAH is likely extremely complex.

Tumor necrosis factor- α (TNF- α) is one of the principal proinflammatory cytokines in the body, and is thought to have a role in the formation and rupture of IAs based on evidence showing significantly increased levels of TNF- α mRNA in IA samples (Jayaraman *et al*, 2005). The *TNF- α* gene exhibits a polymorphism in the promoter region at -308bp, with either guanine (G) or adenine (A) at that location. In a recent study, Fontanella *et al* (2007a) investigated the role of the *TNF- α* gene in aSAH. They examined 171 consecutive patients with aSAH, as well as 144 healthy controls, and showed that the G allele was significantly more frequent in aSAH patients compared with controls, with individuals homozygous for the G-allele having a twofold increase in the risk for developing aSAH. Interestingly, homozygotes for the G allele produced decreased levels of the active cytokine, thus the mechanism by which TNF- α influences the risk of aSAH remains

Table 2 Genetics studies of clinical outcome after aSAH

Insulin-like growth factor-1 (IGF-1)	Ruigrok <i>et al</i> (2005) ^a	Patients carrying an IGF-1 non-wild-type allele of the variable CA repeat (allele with 19 CA repeats is wild type) had a lower risk of poor outcome (3-month GOS score) after aSAH
Interleukin-1 α (IL-1 α)	Ruigrok <i>et al</i> (2005)	The 889 C-T SNP is not associated with outcome (3-month GOS score) after aSAH
Interleukin-1 β (IL-1 β)	Ruigrok <i>et al</i> (2005)	The 511 C-A SNP is not associated with outcome (3 month GOS score) after aSAH
Interleukin-6 (IL-6)	Ruigrok <i>et al</i> (2005)	The 174 G-C SNP is not associated with outcome (3 month GOS score) after aSAH
Tumor necrosis factor- α (TNF- α)	Ruigrok <i>et al</i> (2005) ^a	TNF- α non-WT of the -863 C/A SNP associated with increased risk of poor outcome (3-month GOS score) after aSAH
Brain-derived neurotrophic factor (BDNF)	Vilkki <i>et al</i> (2008) ^a	The BDNF Val66Met SNP was not associated with learning and memory performance. In patients without cerebral infarction, Met carriers had inferior learning and memory performance after aSAH
Apolipoprotein E (ApoE)	Gallek <i>et al</i> (2009) ^a	ApoE ϵ 4 allele is associated with poor outcome (3- and 6-month GOS/MRS scores) after aSAH
	Juvela <i>et al</i> (2009)	No association of ApoE ϵ 2 or ϵ 4 alleles with outcome (GOS score) after aSAH
	Lanterna <i>et al</i> (2007) ^a	Expression of the ϵ 4 allele is associated with a higher risk of a negative outcome (GOS, RDI, and MMSE scores) and delayed ischemia after aSAH
	Louko <i>et al</i> (2006) ^a	ApoE ϵ 4 allele poses a minor risk for late cognitive impairment after the subacute phase of aSAH
	Martinez-Gonzalez <i>et al</i> (2006) ^a	ApoE ϵ 4 genotypes were associated with increased death or dependency after aSAH
	Lanterna <i>et al</i> (2005) ^a	ApoE ϵ 4 allele negatively affects cognitive morbidity and delayed ischemic neurologic deficit after aSAH
	Ruigrok <i>et al</i> (2005)	No association of ApoE ϵ 2 and ϵ 4 alleles with outcome (3-month GOS score) after aSAH
	Morris <i>et al</i> (2004)	No association of the ApoE ϵ 4 and neuropsychological outcome after aSAH
	Tang <i>et al</i> (2003) ^a	Patients with ApoE ϵ 4 allele were predisposed to unfavorable outcomes (3-month GOS score) after aSAH
	Leung <i>et al</i> (2002) ^a	ApoE ϵ 4 genotype is related to poor outcome (6-month GOS score) in aSAH patients
	Niskakangas <i>et al</i> (2001) ^a	ApoE ϵ 4 polymorphism is related to poor outcome (follow-up GOS score) after aSAH
	Dunn <i>et al</i> (2001)	No association of the ApoE ϵ 4 polymorphism with poor outcome (6-month GOS score) after aSAH

aSAH, aneurysmal subarachnoid hemorrhage; GOS, Glasgow Outcome Score; MMSE, Mini Mental Status Exam; MRS, modified Rankin Scale; SAH, subarachnoid hemorrhage; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats; WT, wild type.

^aStatistically significant differences in outcome in the study.

unclear. Despite this association between TNF- α and aneurysmal rupture, TNF- α genotype did not significantly affect the clinical features or outcome of the disease, including the presence or absence of ischemia secondary to vasospasm during the 6-month follow-up period. This may be related, in part, to the multiplicity of actions of TNF- α , depending on the temporal and cell-specific nature of its localization (Shohami *et al*, 1999).

Investigators have also examined whether functional polymorphisms of a number of ischemia-related genes including insulin-like growth factor-1 (IGF-1), TNF- α , interleukin 1- α (IL-1 α), IL-1 β , and IL-6 influence outcome after aSAH (Ruigrok *et al*, 2005). One study analyzed 167 consecutive aSAH patients and used logistic regression with adjustment for known prognostic factors for outcome, assessed by GOS at approximately 3 months after aSAH. Patients carrying an IGF-1 non-wild-type allele had a decreased risk of poor outcome (OR 0.4, 95% CI 0.2 to 1.0), whereas patients carrying the TNF- α non-wild-type allele

had an increased risk (OR 2.3, 95% CI 1.0 to 5.4). In other words, contrary to the results of Jayaraman *et al* (2005), patients with lower serum TNF- α concentrations exhibited a higher frequency of poor outcome after aSAH. No association with outcome was shown for IL-1 α , IL-1 β , or IL-6. This study again highlights the likelihood of multiple, complex roles of inflammatory genes in mediating recovery from ischemic cerebral injury in the setting of aSAH.

Although the previously discussed studies were mainly focused on identifying injurious gene polymorphisms and mechanisms, another approach is to investigate genes thought to be important for the process of functional recovery after aSAH. These genes include those coding for various growth factors, such as brain-derived neurotrophic factor (BDNF), that are known to be upregulated in the context of cerebral inflammation and have a role in recovery in other disease states. In a recent study, Vilkki *et al* (2008) tested 96 patients using a battery of neuropsychometric tests 1 year after aSAH. Genotyping was

performed for the BDNF Val66Met polymorphism. In the total cohort studied, there were no differences in test scores based on this polymorphism, but among patients without cerebral infarction Met carriers had inferior learning and memory performance compared with Val/Val homozygotes. These two groups did not differ on nonmemory test performances, however. The authors suspected that patients with left and bilateral infarctions had deficits in verbal memory that might have confounded the results. Despite some glaring design flaws including the lack of normative data to provide controls for their psychologic testing, this study lends support to the idea of a genetic influence on cognitive recovery after aSAH, which is worth exploring in future studies.

Apolipoprotein E: A myriad of studies have been performed over the past decade seeking to identify associations between apolipoprotein E (ApoE) and outcome after aSAH (Table 2). ApoE is a polymorphic protein that is associated with plasma lipoproteins and is involved in the metabolism and transport of lipids in the central nervous system. Three common alleles of the *ApoE* gene are found in humans ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) on chromosome 19p13, each of which encodes a different form of the protein that varies by only one amino acid. One of these alleles, ApoE $\epsilon 4$, has been associated with increased risk for Alzheimer's disease as well as poor outcome after traumatic or hemorrhagic brain injury (Alberts *et al*, 1995; Fontanella *et al*, 2007b; Teasdale *et al*, 1997). Although the specific molecular basis for this increased risk is unknown, it is suspected that ApoE is critical for the maintenance and repair of neuronal membranes, as well as the regulation of synaptic remodeling after brain injury (Mahley, 1988; Poirier, 1994). This is supported by data from rodent models of head injury that show that ApoE influences neuronal repair, regeneration, and survival (Graham *et al*, 1999; Horsburgh *et al*, 2000). In fact, in an experimental model of SAH, mice expressing the $\epsilon 4$ protein had greater functional deficit, mortality, and vasospasm after induced SAH compared with mice with the $\epsilon 3$ protein (Gao *et al*, 2006). Interestingly, in large population-based studies, despite an association with poor outcome after hemorrhagic and traumatic brain injury, the $\epsilon 4$ allele has not been shown to correlate similarly with poor outcome after ischemic stroke (McCarron *et al*, 1998; McCarron *et al*, 2000).

The first genetic association study examining the association of ApoE alleles with aSAH included 126 patients and was performed in 2001 (Niskakangas *et al*, 2001). Apolipoprotein E genotype and outcome data (GOS) on follow-up examination (the specific time point was undefined) were examined in 86 patients for whom the data were available. In this study, ApoE $\epsilon 4$ genotype was strongly associated with unfavorable outcome (GOS score) after adjustment for age, rebleeding, clinical status on admission, and Fisher grade (OR 7.1, 95% CI 1.9 to 26.3; $P=0.0035$). A second study subsequently followed 96 patients with aSAH admitted to hospital for a period of 6 months, but the authors were unable to detect a significant association between the presence of the ApoE allele and worse clinical outcome (GOS score at 6 months) (Dunn

et al, 2001). However, they did note that those patients who reached the hospital had a lower incidence of the $\epsilon 4$ allele than the general population, suggesting that there is a possibility of increased early mortality in aSAH patients with the $\epsilon 4$ allele. Small sample size and coarse outcome measurements (GOS) might have reduced the power needed to discern subtle differences in outcome. In the largest study of ApoE in aSAH to date, Gallek *et al* (2009) genotyped 206 aneurysmal patients for ApoE polymorphisms and the outcome was determined using GOS and modified Rankin Scale (mRS) at 3 and 6 months after rupture. The authors found no significant differences between cohorts, about the $\epsilon 4$ allele and outcome alone. However, when controlling for cerebral vasospasm as well as the covariates listed above, patients with the $\epsilon 4$ allele had worse functional outcomes at both time points. This suggests that although the $\epsilon 4$ allele may not influence the development of vasospasm, it may impact neuronal response to cerebral ischemia, with ineffective membrane repair leading to poorer outcomes. The most recent study prospectively examined whether the ApoE $\epsilon 2$ or $\epsilon 4$ alleles affected outcome (GOS score) after aSAH in 105 patients (Juvela *et al*, 2009). The authors found that neither allele was associated with outcome or infarction in either univariate or multivariate analyses, leading them to conclude that ApoE polymorphisms have no prognostic value for outcome after aSAH. This conclusion differs from previous studies in aSAH, but is in concordance to previous studies focusing on ischemic stroke (McCarron *et al*, 1999).

A more subtle effect of the ApoE $\epsilon 4$ allele on outcome after aSAH may be revealed through the use of more sensitive outcome markers, such as neuropsychometric tests. Morris *et al* (2004) investigated whether the ApoE $\epsilon 4$ genotype was associated with cognitive and emotional outcome at a mean of 16 months after aSAH. There was no consistent association between the presence of the $\epsilon 4$ allele and performance on neuropsychometric tests, GOS, or SF-36. Lanterna *et al* (2005) also studied 101 noncomatose patients admitted to their institution between 1996 and 2003, with 26 patients found to have the $\epsilon 4$ allele. The presence of the $\epsilon 4$ allele negatively affected overall outcome, as assessed using the Rankin Disability Index and the Mini-Mental Status Exam at 6 months after SAH ($P=0.0087$). Patients with the $\epsilon 4$ allele also experienced more frequent DIND ($P=0.024$) and were more likely to have permanent neurologic deficits ($P=0.0051$). The authors concluded that ApoE $\epsilon 4$ negatively affects cognitive morbidity and DIND resulting from clinical vasospasm after SAH. Limiting their evaluation to noncomatose patients facilitated the use of more complex neurocognitive evaluation, which may be of relevance to the mechanism of ApoE in neurologic recovery after SAH. A smaller study with long-term (12 to 15 years) follow-up showed impairment in visual memory tasks and interference in color naming in aSAH patients who are $\epsilon 4$ carriers, leading the authors to conclude that this allele poses only a minor risk for late cognitive impairment (Louko *et al*, 2006).

In contrast, other studies suggest that the *ApoE* gene constitutes a disease-modifier gene, and that patients with an ApoE $\epsilon 4$ allele are more sensitive to the neuronal and

oxidative damage induced by SAH in the acute phase. One such study showed that patients with the $\epsilon 4$ allele had significantly higher Hunt–Hess grades on admission ($P=0.0014$; Fontanella *et al*, 2007b), however there was no significant association between ApoE allele and outcome (6-month GOS score) in this cohort.

Two studies focusing specifically on Asians were conducted and reached similar conclusions. Leung (2002) showed a significant association between the ApoE $\epsilon 4$ allele and poor outcome (GOS score) at 6 months in a multivariate model (OR 11.3, 95% CI 2.2 to 57.0; $P=0.003$). Tang *et al* (2003) subsequently examined a Chinese population of 104 patients with aSAH and confirmed this association, but went further by showing that patients with unfavorable outcomes (3-month GOS score) had higher levels of serum low-density lipoprotein, total cholesterol, and apolipoprotein B than those with favorable outcomes, suggesting a possible causative mechanism for injury in patients with the $\epsilon 4$ allele. Furthermore, these negative associations were not observed for either the $\epsilon 2$ or $\epsilon 3$ alleles. The importance of this study was that it attempted to establish that the role of ApoE in lipoprotein metabolism has a causative effect on outcome after aSAH. Importantly, the frequency of the $\epsilon 4$ allele varies among different ethnic groups, with a prevalence in the Chinese population between 5% and 8%, much lower than in the average white population, where the prevalence is approximately 20% (Leung *et al*, 2002). The frequency of the $\epsilon 4$ allele in the population studied by Leung *et al* was 21.1%, suggesting a causal link between $\epsilon 4$ allele and aneurysmal rupture. These studies reinforce the notion that the findings of genetics studies that predominantly enroll only one racial or ethnic group may not be generalizable across different populations.

Meta-analyses of ApoE genotype: Given the large number of studies with conflicting results, meta-analysis of the relationship between ApoE genotype and functional outcome after SAH has been performed (Lanterna *et al*, 2007). The authors included eight observational studies, with 696 patients for the analysis of clinical outcome, and 600 patients for the analysis of delayed ischemia. They were able to obtain raw data from the original studies, allowing for analysis of the distribution of baseline variables, quantification of patients lost to follow-up, inclusion of pertinent patients, and better insight into population-stratification bias. In addition, effect sizes were pooled with a random effects model to account for underlying heterogeneity. The authors showed that the risk of a poor outcome (OR 2.558, 95% CI 1.61 to 4.065) as well as delayed ischemia (OR 2.044, 95% CI 1.269 to 3.291) was increased in carriers of the $\epsilon 4$ allele. Poor outcome was defined as death, functional dependency (GOS 2 to 3 or mRS 3 to 5), or severe cognitive impairment (Mini Mental Status Exam score <24). The secondary end point was the development of DCI. Taking into account existing experimental evidence, the authors proposed that ApoE exerts its effects to scavenge free radicals, modulate the inflammatory response, and helps with membrane repair and synaptic plasticity. In addition, individuals with the $\epsilon 4$ allele likely have deficits in these critical functions,

predisposing to poor outcome after cerebral injury. As such, it is likely that ApoE mediates susceptibility to, and recovery from, secondary ischemic injury after aSAH rather than having an integral part in the initial pathogenesis of this disease. The specific mechanism by which ApoE is associated with outcome after aSAH, as well as the functional effect of its various polymorphisms, remains unclear. Further studies are necessary to answer these questions.

Pitfalls of Genetic Association Studies in aSAH

Initial findings of genetic association studies of aSAH have often been difficult to replicate in later studies. Several factors can be identified as reasons why genetic studies in general, and those focusing on aSAH in particular, have encountered these difficulties. The single most common limitation is the small sample size included in these investigations. Aneurysmal SAH is a relatively low-incidence disease, thus the numbers of patients available for enrollment into genetic research protocols is limited, particularly for single-center studies. The negative effect of small sample sizes is compounded by the heterogeneity of the aSAH population in general (i.e., variations in Hunt–Hess grade, Fisher grade, aneurysm location/size, etc.), which further reduces the power of genetic studies to detect clinically significant associations. In addition, when investigations focus on a process that affects only a subset of aSAH patients, such as development of cerebral vasospasm, available subject numbers are reduced even further. The net result is that the majority of small, single-center genetic studies in aSAH are underpowered, increasing the likelihood of type II errors. Negative studies should therefore be viewed critically, particularly when the number of subjects is small or the end point is a relatively low-frequency event (i.e., infarction from vasospasm). For this reason, publication bias is a significant issue in this field. Nevertheless, negative studies, even if underpowered, have the potential to be useful, particularly in providing pilot data that can then be used to design an adequately powered investigation. Dissemination of these results to the scientific community should therefore be encouraged.

Also thought to affect the power of association studies is the type of genetic marker used in the analysis. The majority of aSAH association studies have focused on SNP analysis rather than haplotype analysis, which serves as another possible explanation for inconsistent, sometimes contradictory results seen across multiple studies. A haplotype is a set of genetic markers, as in a set of SNPs, transmitted together on the same chromosome. Haplotype analyses, in some instances, are thought to provide greater statistical power than association studies based on their underlying SNPs (Bader, 2001). An alternative source of error is rooted in the design of studies requiring selection of a control group. Control group selection in these populations is crucial, as any unknown allele variation between cases and controls may appear as disease association. This becomes particularly relevant in eNOS association studies, for example, the majority of which are not

Table 3 Comparison of outcomes evaluated in genetic studies of aSAH

Genes investigated	Author	N	Vasospasm			GOS	DIND	Infarct	mRS	Cognitive function ^a
			Angio	Angio/TCD	Symptomatic					
eNOS T-786C	Khurana <i>et al</i> (2004a,b)	51								
	Song <i>et al</i> (2006)	136								
	Ko <i>et al</i> (2008)	347								
	Starke <i>et al</i> (2008)	77								
PAI-1 4G	Vergouwen <i>et al</i> (2004)	126								
FXIII, PAI-1, TAFI, tPA	Ladenvall <i>et al</i> (2009)	183								
FXIII, FV, MTHFR, Prothrombin	Ruigrok <i>et al</i> (2009)	207								
TNF- α G	Fontanella <i>et al</i> (2007a,b)	171								
ApoE, IGF-1, TNF-A, IL-1B, IL-6	Ruigrok <i>et al</i> (2005)	167								
BDNF	Vilkki <i>et al</i> (2008)	96								
ApoE	Niskakangas <i>et al</i> (2001)	108								
	Dunn <i>et al</i> (2001)	96								
	Tang <i>et al</i> (2003)	104								
	Leung <i>et al</i> (2002)	72								
	Morris <i>et al</i> (2004)	70								
	Lanterna <i>et al</i> (2005)	101								
	Vilkki <i>et al</i> (2008)	46								
	Louko <i>et al</i> (2006)	27								
	Fontanella <i>et al</i> (2007a,b)	146								
	Gallek <i>et al</i> (2009)	206								
	Juvela <i>et al</i> (2009)	105								

Angio, conventional angiography used to assess for vasospasm; Angio/TCD, conventional angiography and transcranial Doppler; symptomatic vasospasm, clinical deterioration attributed to vasospasm; DIND, delayed ischemic neurological deficits, clinical deterioration secondary to delayed ischemia; GOS, Glasgow Outcome Scale; Infarct, presence of a radiographic infarction; mRS, modified Rankin Scale; N, number of patients studied. Shaded boxes indicate that the outcome was evaluated.

^aA variety of cognitive and neuropsychiatric functional tests were used across studies.

matched for ethnicity. Failure to properly match for ethnicity is problematic in light of recent evidence showing striking interethnic disparities in the distribution of eNOS polymorphisms among African Americans and Caucasian Americans (Tanus-Santos *et al*, 2001), as well as black, white, and Amerindian Brazilians (Luizon *et al*, 2009).

Much of the inconsistency in genetic association studies across multiple disease processes may also result from methodological differences between studies (Dichgans and Markus, 2005). Perhaps the greatest impairment to interpreting data from genetic studies is their lack of consensus regarding the definition of clinical events and the use of varying outcomes measures. Widely disparate end points (Table 3) limit the ability to synthesize data from multiple smaller genetic studies of aSAH and perform adequate meta-analyses. For example, the definition of vasospasm may alternatively be defined as angiographic vasospasm, vessel narrowing as detected by transcranial Doppler ultrasound, or on a clinical basis only (DIND) depending on the individual study. In fact, genetic association studies frequently define vasospasm using measures other than conventional angiography (Tables 1 and 3), the gold standard for radiographic confirmation of vasospasm. Aside from lack of uniformity across studies, these alternative definitions preclude a conclusive determination regarding genetic susceptibility to radiographic vasospasm because of their relative lack of sensitivity. Transcranial Doppler ultrasound, for example, was determined in a meta-analysis as approximately 67% sensitive

for middle cerebral artery spasm and 42% sensitive for anterior cerebral artery spasm (Lysakowski *et al*, 2001).

Functional outcome has been assessed using an assortment of grading scales at variable time points, including the mRS, GOS, extended Glasgow Outcome Scale (GOS-E), and neuropsychometric scales. This is compounded by the fact that neurologic outcome is complex, making definition of target phenotypes essential. For instance, many genetic studies have appropriately distinguished between stroke subtypes in large studies (i.e., aSAH versus ICH), however this also significantly decreases the number of available subjects for study. Studies that use more sophisticated outcome measures targeting specific domains of neurologic recovery may more successfully uncover genetic associations.

Although type II error is arguably the greatest limitation of genetic studies of aSAH, positive associations should likewise be viewed critically, as contributions to the observed effect by other factors, both clinical and genetic, may remain uncharacterized. In addition, multiple testing increases the possibility of false-positive associations if appropriate corrective statistics are not used, such as the Bonferroni procedure (Zou and Zuo, 2006). Moving forward, large, multicenter, prospective studies that are able to enroll greater numbers of subjects and investigate the effect of multiple genes/polymorphisms simultaneously and combine these results in a multivariate model are likely to provide the most robust data. As such, collaboration and sharing of data between investigators and research centers is paramount and should be fostered.

Conclusion

Increasing evidence points to a genetic role in the pathophysiology of the ensuing vascular response with development of cerebral vasospasm, occurrence of DCI, and impairment of functional outcome after aSAH. These studies suggest that the mechanisms of injury after aSAH may result from an association of varied gene products throughout the progression of the disease process. However, given the inconsistencies observed between many of these emerging studies, researchers must interpret data and conclusions with caution, and it is essential to reproduce previous results, particularly for smaller genetic studies. Large, multicenter genetic studies involving collaborations between several tertiary care centers will be required to provide the most rigorous results. Moving forward, investigators designing both small- and large-scale genetic association studies should determine consensus outcome definitions and relevant outcome selection early in the course of disease study. Uniformity in study design will allow for more informative results, and will enable investigators to pool data in the event of conflicting results and inadequate power. Although further research is needed, insight into the genetic determinates of outcome after aSAH will help to unravel the complex pathophysiology of this disease and ultimately translate into improved care for these patients.

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Disclosure/conflict of interest

The authors declare no conflicts of interest.

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