Association between genetic variation on chromosome 9p21 and aneurysmal subarachnoid haemorrhage

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ABSTRACT

Background and aim*: Genetic factors play a role in susceptibility to subarachnoid haemorrhage, but little is known about which genes are involved. Recently genome-wide association studies have identified the 9p21 region as a risk locus for intracranial aneurysms (IA). The aim of the present study was to examine the possible association between 9p21 and ruptured IA, ie aneurysmal subarachnoid haemorrhage (aSAH), in a Swedish population. There is one study showing association between 9p21 and arterial stiffness, and arterial stiffness plays a role in the development of hypertension. Therefore a second aim was to investigate whether a putative association is independent of hypertension.*

Methods*: The study comprises 183 patients presenting with aSAH at the Neurointensive Care Unit at the Sahlgrenska University Hospital and 366 healthy age and sex matched population-based controls. As the causative functional variant in the region not yet has been identified, a 44 kbp region on 9p21 was tagged using HapMap. Six SNPs were genotyped.*

Results*: Two SNPs, rs10757278 and rs1333045, showed significant associations with aSAH in univariate analyses. After adjustment for hypertension as well as smoking, the association between aSAH and rs10757278 remained significant with an odds ratio for aSAH of 1.42 (95% CI 1.08-1.87, p=0.01) for the uncommon G allele.*

Conclusions*: We confirm earlier results showing that 9p21 is a susceptibility locus for IA, and we show that this association is present in a Swedish sample restricted to ruptured IA. For the first time we demonstrate that this association is independent of hypertension.*

INTRODUCTION

Subarachnoid haemorrhage (SAH) is a serious condition that has a young age onset and poor outcome. The incident rate in Sweden is 12.4/100 000 person-years.[1] SAH is in 85% of the cases caused by the rupture of an intracranial aneurysm (IA).[2] The most important risk factors are smoking, hypertension, and excessive alcohol intake.[3] SAH occurs more often in women.[4] In addition, genetic factors have also shown to play an important role in SAH. First-degree relatives of SAH patients have three to five times higher risk of SAH than the general population.[5] Furthermore, it is estimated that about 9% of first degree relatives to patients suffering from SAH/IA harbour an unruptured IA,[6, 7] whereas the population frequency is approximately 2%.[8]

As there is heritability in IA, whole-genome linkage studies have been conducted and linkage has been found to several loci. In the largest whole-genome linkage study comprising 333 families possible linkage to 4q and 12p was detected [9]. There is also one genome-wide association study (GWAS) on IA, on two European populations and one Japanese population, in which associations were found for 2q33, 8q11 and 9p21 [10]. Association between 9p21 and IA was also shown by Helgadottir et al in a candidate gene study including three different European populations [17]. Interestingly, 9p21 also shows association to other vascular phenotypes such as coronary artery disease (CAD),[11-15] ischemic stroke (IS),[16] aortic aneurysms,[17] and arterial stiffness.[18] The molecular mechanism underlying these associations has not yet been identified. However, the findings that 9p21 is associated with aneurysms and arterial stiffness suggest that genetic variations at this locus may influence vessel properties, thereby leading various vascular diseases susceptibility.

The aim of the present study was to investigate whether there is an association between genetic variants on 9p21 and ruptured IA, ie aneurysmal subarachnoid haemorrhage (aSAH), in a Swedish population, and if so, to evaluate whether this association is independent of hypertension as well as smoking.

METHODS

Study Population

Consecutive Caucasian patients presenting with SAH admitted to the Neurointensive Care Unit (NICU) at the Sahlgrenska University Hospital, between 2000 and 2004, were recruited. During this period, out of 253 patients considered, 183 fulfilled the diagnostic criteria and were willing to participate in the study. A detailed description of the inclusion criteria has been published.[19, 20] SAH was defined by symptoms suggestive of SAH combined with subarachnoid blood on computed tomography (CT). Only patients with aSAH, defined as an aneurysm visualised on intra-arterial angiography, were included. Patients were treated with neurosurgical clipping or by endovascular coiling. For each case two healthy Caucasian controls, matched for age (+/-2 years), sex, and geographical residence area, were randomly selected from a population based health survey[21] or from the Swedish Population Register. The latter source was used to recruit controls younger than 30 years of age. All participants gave informed consent, and when patients were unable to communicate his or her next-of-kin gave informed consent. The Medical Ethics Committee at the University of Gothenburg approved the study.

Clinical characteristics

Smoking history was coded as current versus never or former smoker (smoking cessation at least one year before inclusion in the study) and hypertension was defined by pharmacological treatment for hypertension prior to the event.

Genotyping

Because the causative functional variant(s) in the region 9p21 has not yet been identified, a region of 44kbp, between position 22071397 and 22115503, on 9p21 was tagged using HapMap CEU data (Rel 23a) and HaploView 4.1 (r^2=0.8 and minor allele frequency (MAF) 0.1), resulting in 6 tag SNPs, rs10965227, rs1547705, rs7857345, rs1333045, rs1333040, and rs10757278. The analysed region includes SNPs that in previous studies have been shown to associate to IA (figure 1).[10, 17] The SNP rs1537378, recently found to be associated to the ischemic stroke subtype large vessel disease (LVD), was also included.[22] Genotyping was performed by TaqMan Assays. Primers and probes were obtained from Applied Biosystems (C_31288976_10, C_1754687_20, C_29146326_10, C_8766826_10, (C_8766795_10), C_11841860_10, and C_83169_10). Amplifications were carried out on a 384 Well GeneAmp® PCR System 9700 (Applied Biosystems) and fluorescence was analysed on an ABI PRISM® 7900HT Sequence Detector System (Applied Biosystems). Genotyping was performed blinded to case-control status. The assay for rs1333040 (C_8766795_10) failed and could not be replaced. The five SNPs that were successfully genotyped tag 90% of the genotyped SNPs in HapMap, in the region of interest.

Statistics

Allele frequencies were derived from genotype data, and deviations from the Hardy Weinberg equilibrium (HWE) were tested. Associations between single SNPs and case control status were investigated using univariate and multivariate binary logistic regression using an additive model. The multivariate model included hypertension and smoking status as covariates. The interaction between genotype and smoking/gender was also tested in a binary logistic regression. Haplotype frequencies and associations between haplotypes and case controls status were estimated using THESIAS.[23] This method uses a stochastic EM algorithm for likelihood maximisation. The individual likelihood for each haplotype was used to estimate odds ratios (ORs) for each haplotype by comparison to a reference haplotype represented by the most frequent haplotype. Data was analysed using SPSS 16.0 (SPSS Inc, USA), and statistical analyses were performed in a two tailed fashion. P<0.05 was considered significant. The studied SNPs have MAFs between 0.14 and 0.50 (median 0.30) according to HapMap (CEU population), and it was estimated that the study has 80% power to detect an OR of 1.45 at the 5% level for SNPs with a MAF of 0.3 for the risk allele. No correction for multiple testing was conducted since most SNPs are in high LD and a Bonferroni correction would be too conservative.

Missing values

Information on smoking status was missing in six cases due to intervening death, and information on hypertension was missing in one case. Genotyping success rates were between 98 and 100%.

RESULTS

Clinical characteristics for this sample have been published.[19] In short, the mean age of patients and controls was 55 (range 20–81) years and 74% were females. As expected, smokers were more frequent in cases compared to controls (54% versus 20%). Furthermore, 23% of patients and 19% of controls had antihypertensive treatment. Genotype frequencies are presented in table 1. In controls all genotype frequencies were consistent with HWE.

Two SNPs showed a significant association with aSAH in univariate logistic regression analysis (table 2). The uncommon allele (G) of SNP rs10757278 was associated with an increased risk of aSAH. This association remained

significant after adjustment for hypertension and smoking (table 2). For comparative purpose with earlier data, ORs for both heterozygote and heterozygote subjects were calculated. The univariate ORs for aSAH were 1.25 (95% CI 0.80-1.95) and 1.95 (1.18-3.22) for heterozygote subjects and for subjects homozygote for the rs10757278 G allele, respectively. The corresponding multivariate ORs were 1.38 (95% CI 0.85-2.24) and 2.01 (1.16-3.49), respectively. Approximately 24% of the patients and controls were homozygous for the rs10757278 G allele, which is in line with previous studies on individuals of European descent.[17] The uncommon allele (A) of rs1333045 was associated with a decreased risk of aSAH (table 2). However, this association did not remain significant after adjustment for hypertension and smoking (table 2). In the logistic regression analysis an additive model was used. Because the causative variant is unknown, the genetic effect remains to be determined. In addition, we therefore compared genotype frequencies in cases and controls by the chi-square test. For the lead SNP, rs10757278, there was also a significant difference with regard to genotype frequencies between cases and controls (table 1). Haplotype analysis, including SNP rs10965227, rs1547705, rs7857345, rs1333045, and rs10757278, did not add any further information compared to single SNP analyses (data not shown).

Because rs10757278 was the lead SNP in the overall analysis, we focused on this variant in subsequent subanalyses. In a sex specific subanalysis no significant sex specific difference was detected. Multivariate ORs of aSAH for rs10757278 were 1.49 (95% CI 1.07-2.07, p=0.02) and 1.27 (95% CI 0.74-2.16, p=0.39) for women and men, respectively.

Smoking is considered to be the most important modifiable risk factor for aSAH,[3] and evidence of an interaction between current smoking and familial aggregation of IA/SAH has been reported.[24] In our sample, 371 participants (79 patients and 292 controls) were non-smokers and 172 (98 patients and 74 controls) were smokers. An association between aSAH and rs10757278 was found in non-smokers, but not in smokers (univariate OR 1.47, 95% CI 1.03-2.10, p=0.03 and OR 1.37, 95% CI 0.89-2.11, p=0.15 respectively). However, there was no significant interaction between genotype and smoking status, i.e. there was no significant difference in the effect of rs10757278 between non-smokers and smokers.

Genotype frequencies for the additional SNP, rs1537378, are presented in table 1. In controls the genotype distribution was in HWE. No significant association with aSAH was detected (table 2). This SNP was not included in the haplotype analysis, since it is located in another linkage disequilibrium (LD) block compared to the other SNPs in this study.

DISCUSSION

This case-control study with participants from Sweden provides evidence for an association between genetic variations on chromosome 9p21 and ruptured IA, thus confirming earlier data on an association between genetic variants on this genetic region and IA.[10, 17] In addition, this association was shown to be independent of hypertension as well as smoking.

The 9p21 candidate region investigated in this study is 44kbp. In addition to the SNPs showing association to IA in previous studies, this region also harbours SNPs that have shown to associate with CAD in several GWAS.[11-15] We found the rs10757278 G allele to be significantly associated with an increased risk of aSAH. The effect of the G allele was additive with univariate ORs of 1.25 and 1.95 for heterozygote and homozygote subjects, respectively. This finding is in line with

results from a recent study on three different populations from northern Europe with different vascular phenotypes, including IA and abdominal aortic aneurysms (AAA).[17] With regard to the phenotype IA, patient inclusion in the mentioned study was based on the diagnosis of IA, but approximately 87% of the patients had suffered from aSAH. The ORs for IA in all three populations combined were 1.38 (95% CI 1.18-1.63) and 1.72 (1.39-2.13) for subjects who were heterozygote and homozygote for the G allele, respectively. A similar effect of the G allele was observed with regard to the risk of AAA.[17] Peripheral artery disease and stroke (large artery atherosclerotic or cardiogenic stroke) also showed significant association with rs10757278 in this study, but with lower ORs.[17]

There is one additional study showing association between 9p21 and IA.[10] The Caucasian populations in this study were from Finland and the Netherlands and in the two cohorts approximately 81% of the patients had suffered from aSAH. Associations to IA were observed for three SNPs on 9p21, rs1333040, rs10116277, and rs2383207, with ORs of 1.58-1.66 for homozygote subjects. As the lead SNP in the present study, rs10757278, is in LD with rs1333040, rs10116277, and rs2383207 (r^2 =0.57, 0.89, and 0.86, respectively) our results are in agreement with the study conducted by Bilguvar et al.[10]

As there is one study showing association between variants on 9p21 and arterial stiffness, and there is data indicating that arterial stiffness is involved in the development of hypertension, [25, 26] it may be important to adjust for hypertension when assessing the association between 9p21 and ruptured IA. We found the association between aSAH and the SNP rs10757278 to be independent of hypertension and also of smoking. This is in line with some studies on other vascular phenotypes that report independent association between 9p21 and IS,[22, 27] the IS subtype LVD,[22, 28] CAD,[14] and myocardial infarction (MI).[13, 14] There are however, conflicting data from two studies that observed associations between genetic variations on 9p21 and IS or MI, that did not remain significant after adjustment for vascular risk factors.[16, 29] However, the latter studies had smaller sample sizes.

Interestingly, there is evidence of an interaction between familial aggregation of IA/SAH and current smoking.[24] However, we did not find any significant interaction between rs10757278 and smoking. Similar findings could be seen for CAD.[15] Thus, the interaction between family history and smoking is likely to be mediated by other genes. In fact, preliminary data from a GWAS on Caucasian families from the United States, show a multiplicative interaction between smoking and SNPs in the phosphodiesterase 1A (PDE1A) and type IX collagen (COL9A1) genes for IA susceptibility.[30]

We also investigated rs1537378, which is located outside the previously analysed LD block on 9p21, because this SNP was recently reported to show association with the ischemic stroke subtype LVD.[22] In contrast to LVD, we did not detect an association to aSAH.

The 9p21 locus harbouring rs10757278 does not contain any protein coding genes, but a large non-coding RNA (ncRNA) ANRIL. As for most ncRNAs, there is limited knowledge about the function of ANRIL, but expression of ANRIL has been detected in a wide range of cell types and tissues, including AAAs,[15] and ANRIL was suggested as the prime 9p21 candidate gene for CAD.[15] Furthermore, studies indicate that genetic variations on 9p21 influence the expression of ANRIL.[31-33] Potentially interesting

genes adjacent to the investigated region are CDKN2A and CDKN2B. CDKN2A encodes two proteins, p14 (ARF) and p16 (INK4a). CDKN2B encodes p15 (INK4b). These are all tumour suppressor proteins involved in proliferation, aging, and senescence.[34] It has also been proposed that p16 can modulate the production of inflammatory molecules such as matrix metalloproteinase 3 (MMP-3) and monocyte chemoattractant protein 1 (MCP-1).[35] Additionally, expression of ANRIL is positively correlated with the expression of p14, and possibly also with that of p16 and p15, in both physiologic and pathologic conditions suggesting a coordinated transcriptional regulation of theses genes.[36] Further work is needed to determine if the association between 9p21 and aSAH is mediated through ANRIL and/or any of the mentioned genes. Moreover, it can not be excluded that the SNPs of interest could be situated in a distant enhancer effecting genes further away.

This study has some limitations worth noting. Although patients were recruited consecutively, there is of course a recruitment bias due to early fatality as well as due to the fact that patients with initial signs of an unfavourable prognosis often are treated outside the NICU. The inclusion criteria being aSAH also introduced some bias because some patients with poor prognosis do not undergo angiography. The control group was recruited by random sampling from the general population in the same geographical areas as the patients, which makes the possibility of selection bias in this group less likely. In the present study, we controlled for the risk factors hypertension and smoking. However, we did not control for alcohol intake, because it is difficult to obtain reliable data on this variable. The main advantages of our study are the relatively homogeneous and large patient group with confirmed ruptured aneurysms, a population based control group, and a well characterised sample with regards to the risk factors hypertension and smoking.

In conclusion, this study shows a significant association between genetic variation on chromosome 9p21 and aSAH with rs10757278 as the lead SNP. This association was independent of hypertension and smoking.

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Competing Interest:

None declared.

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Table 1 Genotype frequencies

		Control		aSAH		p-value
rs10965227	AA, n (%)	232	(64)	128	(70)	
	AG, n (%)	117	(32)	44	(24)	0.14
	GG, n (%)	15	(4)	10	(6)	
rs1547705	AA, n (%)	290	(79)	137	(75)	
	AC, n (%)	72	(20)	42	(23)	0.14
	CC, n (%)	2	(1)	4	(2)	
rs7857345	GG, n (%)	162	(44)	96	(53)	
	GA, n (%)	163	(45)	72	(39)	0.17
	AA, n (%)	41	(11)	15	(8)	
rs1333045	GG, n (%)	91	(25)	60	(33)	
	GA, n (%)	186	(51)	88	(48)	0.10
	AA, n (%)	88	(24)	34	(19)	
rs10757278	AA, n (%)	106	(29)	40	(22)	
	AG, n (%)	184	(50)	87	(47)	0.02
	GG, n (%)	76	(21)	56	(31)	
rs1537378	GG, n (%)	108	(30)	64	(35)	
	GA, n (%)	193	(53)	95	(53)	0.19
	AA, n (%)	63	(17)	22	(12)	

aSAH, aneurysmal subarachnoid haemorrhage. The p-values indicate differences in genotype frequencies between controls and cases as calculated using chi-square test (2df).

Table 2 Association between SNPs on 9p21 and aSAH

SNP	Uncommon allele	Univariate OR (95% CI)	Multivariate* OR (95% CI)
rs10965227	G	0.85 (0.62-1.17), p=0.32	0.83 (0.58-1.19), p=0.31
rs1547705	C	1.37 (0.93-2.01), p=0.11	1.39 (0.91-2.11), p=0.12
rs7857345	A	0.77 (0.58-1.01), p=0.06	0.80 (0.59-1.07), p=0.14
rs1333045	A	0.76 (0.59-0.98), p=0.04	0.78 (0.59-1.02), p=0.07
rs10757278	G	1.40 (1.09-1.80), p=0.01	1.42 (1.08-1.87), p=0.01
rs1537378	A	0.78 (0.60-1.02), p=0.07	0.8 (0.60-1.07), p=0.14

aSAH, aneurysmal subarachnoid haemorrhage
OR, odds ratio
CI, confidence interval
ORs were calculated using an additive model
*Multivariate analysis adjusted for hypertension and smoking status

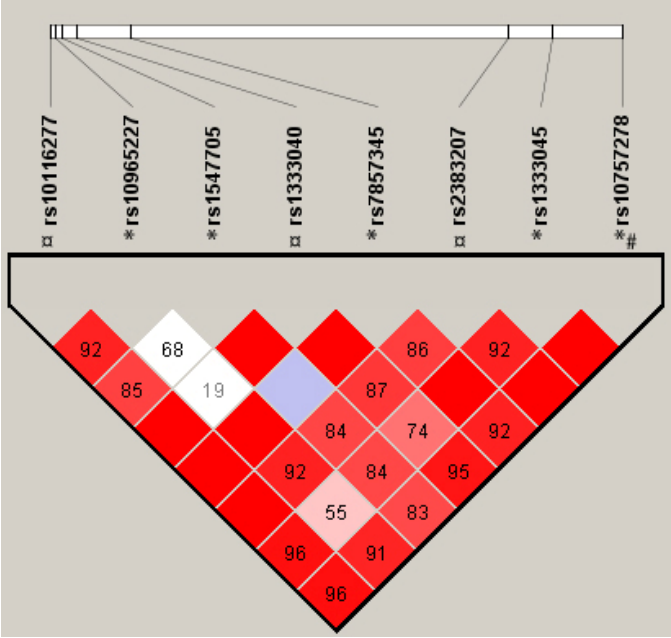

Figure 1 Graphic representation of the LD structure in the region 9p21 between position 22071397 and 22115503 (NCBI build 36) downloaded from the HapMap database (www.hapmap.org) for the CEU population. This plot shows SNPs assayed in the present study, and SNPs previously associated with IA. * SNPs analyzed in the present study; ¤ SNPs analyzed by Bilguvar et al [10]; # SNP analysed by Helgadottir et al [17]