Polymorphisms of Homocysteine Metabolism Are Associated with Intracranial Aneurysms

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Key Words

Homocysteine • Intracranial aneurysm • Polymorphism • Transcobalamin 2

Abstract

Background: Impaired homocysteine metabolism is associated with a number of vasculopathies including extracranial aneurysms. We analyzed the possible association of nine genetic variants of homocysteine metabolism with the occurrence of intracranial aneurysms. **Methods:** Caucasian patients (n = 255) treated at two German hospitals for intracranial aneurysms and local controls (n = 348) were genotyped for the following polymorphisms: methionine synthase (MTR) c.2756A→G, methylenetetrahydrofolate reductase (MTHFR) c.677C→T, MTHFR c.1298A→C, cystathionine β-synthase (CBS) c.844_855ins68, CBS c.833T→C, dihydrofolate reductase (DHFR) c.594 + 59del19bp, glutathione S-transferase Ω-1 (GSTO1) c.428C→A, reduced folate carrier 1 (RFC1) c.80G→A and transcobalamin 2 (Tc2) c.776C→G. **Results:** The G-allele of the missense polymorphism Tc2 c.776C→G was found to be underrepresented in patients, suggesting that this variant may protect from the formation of cerebral aneurysms [odds ratio per two risk alleles (OR) 0.48; 95% confidence interval (CI) 0.30–0.77; p = 0.002]. We obtained borderline results for the G-allele of RFC1 c.80G→A

(OR 1.64; 95% CI 1.01–2.65; p = 0.051) and the insertion allele of DHFR c.594 + 59del19bp (OR 1.61; 95% CI 1.00–2.60; p = 0.059), which were found to be overrepresented in patients. **Conclusion:** Polymorphisms of homocysteine metabolism are possible risk factors for the formation of intracranial aneurysms.

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Introduction

Aneurysmal subarachnoid hemorrhage caused by ruptured intracranial aneurysms has a 30-day mortality rate of up to 45%, and 30% of survivors have moderate-to-severe disability [1]. Little is known about the etiology of intracranial aneurysms. Hereditary factors are supposed to be involved in the etiology of cerebral aneurysms. In addition to defined diseases like autosomal dominant polycystic kidney disease, multiple genetic susceptibilities are considered to act together in the etiology of subarachnoid hemorrhage [2]. The increase in the familial risk of subarachnoid hemorrhage is approximately fourfold among first-degree relatives [3, 4]. Poly-

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morphisms of genes coding for constituents of vascular walls or involved in angiogenesis and vascular remodeling have been suggested to be associated with the formation of cerebral aneurysms [2]. Polymorphisms affecting homocysteine metabolism have been reported to promote atherosclerosis, ischemic stroke, cervical arterial dissections and abdominal aortic aneurysms [5–7]. In addition, autosomal recessive deficiency in cystathionine- β -synthase (CBS; OMIM 236200), which is involved in homocysteine metabolism, presents concomitant with vascular disease including the formation of aneurysms [8]. The present study aimed to analyze whether polymorphisms affecting homocysteine metabolism are associated with the incidence of intracranial aneurysms.

Patients and Methods

Subjects

Patients of Caucasian origin undergoing microsurgery or endovascular treatment for cerebral aneurysms were recruited from the Departments of Neurosurgery at the University of Bonn and the Technical University of Dresden, Germany. Diagnoses were made by brain imaging, including digital subtraction angiography in all cases. Patients with known autosomal dominant polycystic kidney disease, fibromuscular dysplasia or with additional cerebrovascular dysplasia were excluded [9].

This study was approved by the local ethics committees. Written informed consent was obtained from patients or their legal representatives.

As controls we studied 250 apparently healthy Caucasian residents in the area of Bonn (64% female; mean age 50.4 ± 11.9 years) without a history of cerebrovascular disease and 98 Caucasian healthy anonymous blood donors from the Dresden area. The Bonn area residents were recruited from an ongoing study on cerebrovascular disease. They are healthy partners from patients with vascular events, free from any history or ultrasonic signs of vascular disease [10].

Genotyping

Genomic DNA prepared from peripheral leukocytes was used for genotyping by PCR amplification and restriction analysis of nine genetic variants of homocysteine metabolism including the intronic deletion dihydrofolate reductase (DHFR) c.594 + 59del19bp (affecting the transcript level; GenBank NM_000791.3), the splice alteration cystathionine β -synthase c.844_855ins68 (affecting the transcript level; GenBank S78267.1) and the missense mutations (i.e. leading to amino acid exchanges) methionine synthase (MTR) c.2756A $\rightarrow$ G (p.D919G; rs1805087), methylenetetrahydrofolate reductase (MTHFR) c.677C $\rightarrow$ T (p.A222V; rs1801133) and c.1298A $\rightarrow$ C (p.E429A; rs1801131), CBS c.833T $\rightarrow$ C (p.I278T; rs5742905), glutathione S-transferase Ω -1 (GSTO1) c.428C $\rightarrow$ A (p.A140D; rs4925), reduced folate carrier 1 (RFC1) c.80G $\rightarrow$ A (p.R27H; rs1051266) and transcobalamin 2 (Tc2) c.776C $\rightarrow$ G (p.P259R; rs1801198) [11].

Statistical Analysis

Deviation from and compatibility with the Hardy-Weinberg equilibrium was analyzed using a Monte Carlo χ^2 goodness-of-fit test (C-program available from www.imbs-luebeck.de) [12] and the exact uniformly most powerful equivalence test with $\delta_2 = 2$ and $\delta_1 = 2/3$, respectively (SAS macro using SAS version 9.1) [13]. As primary analysis, exact logistic regressions between genotypes and aneurysms were carried out using the Cochran-Armitage trend test for genotypes and stratification by center. Covariates were available for the samples from Bonn, and adjustments for age and gender were performed using the exact logistic regression framework with trend test coding for genotypes (software StatXact 6). Population stratification can be assumed to be negligible in the present study because we extensively investigated population stratification in the entire German population previously [14]. The global test level was set to 5%. Adjustments for multiple testing were done according to the step-down procedure of Šidak-Holm [15]. Asymptotic 95% confidence intervals (CIs) were computed for genotypes without adjustments for multiple testing for the joint analysis of both centers. Exact CIs at the nominal 95% confidence level were calculated for each center without adjustments for covariates (SAS 9.1).

Power calculations using the available sample were carried out using the two-sided asymptotic Cochran-Armitage test at the global 0.625% test level that already adjusts for the analysis of eight successfully genotyped polymorphisms assuming the observed odds ratios (ORs) and genotype frequencies in the controls (StatXact 6).

Results

We investigated 111 patients (74% female, mean age 51.1 ± 12.0 years) of Caucasian origin undergoing microsurgery or endovascular treatment for cerebral aneurysms at the Department of Neurosurgery, University of Bonn, and 144 Caucasian patients admitted to the Department of Neurosurgery, Technical University of Dresden (63% female, mean age 51.1 ± 12.0 years). Seven further patients had been excluded due to autosomal dominant polycystic kidney disease ($n = 3$), fibromuscular dysplasia ($n = 1$) or other cerebrovascular dysplasia ($n = 3$). In 36% of the cases, more than one intracranial aneurysm was diagnosed.

The GSTO1 c.428C $\rightarrow$ A variant showed deviation from the Hardy-Weinberg equilibrium in the controls of Bonn ($p = 0.02$) and was therefore omitted from further analysis. No other deviations were observed in the control populations.

The genotype distribution of Tc2 c.777C $\rightarrow$ G was significantly different between patients and controls ($p = 0.002$; table 1). We observed borderline significant results for DHFR c.594 + 59del19bp ($p = 0.059$) and for RFC1 c.80G $\rightarrow$ A ($p = 0.051$). Analysis of the other poly-

Table 1. Polymorphisms of homocysteine metabolism and aneurysm formation in Caucasian patients from Bonn (n = 111) and Dresden (n = 144)

Genotype	Patients		Controls		p value	
	n	%	n	%	OR	95% CI
CBS c.833T→C:					p = 1.00	
TT	250	98.8	341	98.6	1.00	
CT	3	1.2	5	1.4	0.98	0.22–4.34
CC	0	0.0	0	0.0	0.96	0.05–18.8
CBS c.844ins68:					p = 0.440	
del/del	216	86.7	306	88.4	1.00	
ins/del	32	12.9	40	11.6	1.26	0.76–2.08
ins/ins	1	0.4	0	0.0	1.58	0.58–4.33
MTHFR c.677C→T:					p = 0.984	
CC	116	46.0	158	45.7	1.00	
CT	109	43.3	152	43.9	0.99	0.77–1.28
TT	27	10.7	36	10.4	0.98	0.59–1.63
MTHFR c.1298A→C:					p = 0.609	
AA	117	46.4	161	46.4	1.00	
AC	113	44.8	150	43.2	0.93	0.71–1.20
CC	22	8.7	36	10.4	0.86	0.51–1.44
MTR c.2756A→G:					p = 0.675	
AA	168	66.4	223	64.3	1.00	
AG	81	32.0	112	32.3	0.92	0.67–1.27
GG	4	1.6	12	3.5	0.85	0.45–1.61
DHFR c.594 + 59del19bp:					p = 0.059	
del/del	35	14.1	78	22.7	1.00	
del/ins	124	49.8	153	44.5	1.27	1.00–1.61
ins/ins	90	36.1	113	32.8	1.61	1.00–2.60
RFC1 c.80G→A:					p = 0.051	
AA	76	31.1	129	38.1	1.00	
AG	118	48.4	154	45.4	1.28	1.01–1.63
GG	50	20.5	56	16.5	1.64	1.01–2.65
Tc2 c.776C→G:					p = 0.002	
CC	97	38.5	96	28.1	1.00	
CG	113	44.8	157	45.9	0.69	0.55–0.88
GG	42	16.7	89	26.0	0.48	0.30–0.77

Numbers of genotypes (n) observed and ratios (%) per polymorphism are depicted. Nominal two-sided p values from an exact logistic regression with center as covariate are shown using the trend test coding for genotypes. Asymptotic CIs were calculated for the joint analysis of both centers.

morphisms did not reveal any significant associations.

Although population stratification can be assumed to be negligible in the present study [14], we performed explorative separate analyses for the Bonn and the Dresden populations and found significant differences for the Tc2 variant in the larger Dresden subgroup (patients, CC: 0.43, CG: 0.41, GG: 0.16; controls, CC: 0.23, CG: 0.51, GG: 0.26; p = 0.003), but not for the smaller Bonn subgroup, although the GG genotype was underrepresented in both patient populations (patients, CC: 0.33, CG: 0.50, GG:

0.17; controls, CC: 0.30, CG: 0.44, GG: 0.26; p = 0.161). Similarly, the distribution of the DHFR genotypes was significantly different between patients and controls in the Dresden population (patients, del/del: 0.14, del/ins: 0.46, ins/ins: 0.41; controls, del/del: 0.27, del/ins: 0.44, ins/ins: 0.29; p = 0.010) but not in the Bonn population (patients, del/del: 0.15, del/ins: 0.55, ins/ins: 0.30; controls, del/del: 0.21, del/ins: 0.45, ins/ins: 0.34; p = 0.809). Concerning the RFC1 polymorphism, the distribution of genotypes was significantly different between patients and controls in the Bonn population (patients, AA: 0.24, AG:

0.53, GG: 0.23; controls, AA: 0.38, AG: 0.46, GG: 0.16; $p = 0.012$) but not in the Dresden population (patients, AA: 0.37, AG: 0.44, GG: 0.19; controls, AA: 0.37, AG: 0.45, GG: 0.18; $p = 0.926$).

The power to detect effects of DHFR and RFC1 was only 51 and 52%, respectively, after adjustments for multiple testing. In contrast, the power to detect the effect of Tc2 c.777C→G was 81% even after adjustments for multiple testing.

None of the polymorphisms investigated was significantly associated with the multiplicity of aneurysms, and no significant association with single or multiple aneurysms was seen in subgroups defined by age (not shown).

Discussion

We analyzed nine functional polymorphisms of methionine metabolism in a total of 255 German patients of Caucasian origin treated for intracranial aneurysms compared with 348 German controls of Caucasian origin. The distribution of genotypes of Tc2 c.776C→G was significantly different between patients and controls. Additionally, we obtained borderline results for the polymorphisms RFC1 c.80G→A and DHFR c.594 + 59del19bp. Subgroup analysis of the Bonn and the Dresden population only proved a significant effect in one of these populations for each of those three polymorphisms. Although the allele and genotype frequencies did not significantly differ between the two control groups (not shown), we cannot exclude that differences in the selection of controls (healthy partners of patients with vascular disease vs. anonymous blood donors) might have contributed to the different results between the Bonn and the Dresden subgroups. Further, within the limits of any association study, we cannot exclude random errors associated with the small numbers in spite of correction for multiple testing. The results observed in our study require confirmation in a further patient cohort.

Previous studies suggested that the G-allele of the Tc2 c.777C→G polymorphism, which was underrepresented in patients with intracranial aneurysms in our study, affects vitamin B₁₂ binding affinity and the ability to transport vitamin B₁₂ into tissues [16–18], leading to a reduced remethylation of homocysteine to methionine by vitamin B₁₂-dependent MTR. An impaired homocysteine metabolism may lead to an accumulation of asymmetric dimethylarginine [19, 20], which is a major endogenous inhibitor of nitric oxide and is strongly predictive of prema-

ture cardiovascular disease and death [21, 22]. It has already been shown that nitric oxide availability is a key requirement for the development of intracranial aneurysms, and intracranial aneurysm formation was prevented by inhibition of nitric oxide synthase (NOS) in a rodent model [23]. The size of intracranial aneurysms is significantly smaller in iNOS^{-/-} mice than in iNOS^{+/+} mice [24]. eNOS polymorphisms have been investigated for associations with intracranial aneurysm formation and rupture [25]. We therefore speculate that the protective effect of the Tc2 c.776G allele found in the present study might in part be explained by decreased transport capacity of vitamin B₁₂ in the blood, leading to increased levels of asymmetric dimethylarginine and thereby more effective NOS inhibition. Also, lower homocysteine remethylation by MTR may lead to a higher homocysteine turnover via CBS and, therefore, to a lower capacity of CBS concerning the second reaction catalyzed by this enzyme, the synthesis of the potent vasodilator H₂S, which may contribute to vascular damage [26].

The biochemical consequences of the variant DHFR c.594 + 59del19bp and the RFC1 c.80G→A polymorphism are less well defined. DHFR converts dihydrofolate into 5,10-methylenetetrahydrofolate, a methyl group shuttle required for the de novo synthesis of nucleic acids as well as the formation of 5-methyltetrahydrofolate by MTHFR. The 19-bp deletion in intron-1 of DHFR has been associated with the risk of spina bifida [27–29]. It supposedly decreases DHFR gene expression [29], and reduced DHFR expression might plausibly lead to reduced availability of folates for remethylation of homocysteine to methionine. Thus, the DHFR variant might speculatively have similar biochemical effects as the Tc2 variant. The extracellular reduced folate carrier RFC1 takes up reduced folates into the cell [30]. The c.80G→A polymorphism has been suggested to affect folate and homocysteine metabolism [31]. The biochemical consequences for intracellular, in particular endothelial, metabolism remain speculative.

In summary, the data of our study suggest that the missense polymorphism Tc2 c.776C→G may be associated with the formation of intracranial aneurysms. In addition, the polymorphisms RFC1 c.80G→A and DHFR c.594 + 59del19bp might also be involved. If these results are confirmed in an independent patient cohort, the biochemical effects of these polymorphisms on the intracranial vasculature should be explored in detail. This may well yield important new insights into the pathogenesis of intracranial aneurysms.

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