Angiotensinogen Promoter B-Haplotype Associated With Cerebral Small Vessel Disease Enhances Basal Transcriptional Activity

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Background and Purpose—Previously, we described the presence of 5 haplotypes (A to E) at the angiotensinogen (AGT) promoter and reported a significant association between the B-haplotype (nucleotide substitutions −6:G→A and −20:A→C compared with the wild-type A-haplotype) and magnetic resonance imaging correlates of cerebral small vessel disease (cSVD). The association was independent of hypertension, suggesting a brain-specific effect of this haplotype. In the current study, we investigated transcriptional activities of the 5 promoter haplotypes in astrocytes, the main source of cerebral AGT, and in hepatocytes, the main source of systemic AGT, as well as determined the evolutionary relatedness of the promoter haplotypes.

Methods—Transcriptional activity depending on the haplotypes and the −6:A and −20:C substitutions was measured in transiently transfected A172 and HepG2 cells. We genotyped 5 new single nucleotide polymorphisms (SNPs) at the AGT gene and measured linkage disequilibrium (LD) among SNPs and the promoter haplotypes. An evolution-based haplotype tree was constructed.

Results—The B-haplotype increased transcriptional activity in both cell types. Its effect was stronger in astrocytes than in hepatocytes (2.4±0.09-fold, P<0.001 versus 1.6±0.06-fold, P=0.014). Importantly, in astrocytes the combination of the −6:A and the −20:C was mandatory for increased activity, whereas in hepatocytes the −20:C on its own was sufficient. Strong LD between the 5 new SNPs and the promoter haplotypes allowed the reconstruction of 9 haplotypes over the AGT gene.

Conclusions—Combination of the −6:A and −20:C substitutions in the B-haplotype may promote the development of cSVD by enhancing cerebral angiotensinogen expression. (Stroke. 2004;35:2592-2597.)

Key Words: angiotensinogen • gene expression regulation • genetics • white matter

Cerebral small-vessel disease (cSVD) is an important cause of stroke, cognitive decline, and dementia.1–2 cSVD can be visualized by magnetic resonance imaging of the brain as early confluent and confluent white matter hyperintensities and lacunar infarcts. The estimated heritability of cSVD is 73%, suggesting that genetic factors play an important causative role.3 Arterial hypertension is the major risk factor for cSVD, which also has a high heritability in most populations.4

The renin-angiotensin system (RAS) is an important regulator of blood pressure. Plasma angiotensinogen (AGT) synthesized by the liver is processed to angiotensin II (AT-II) by the serial action of renin and angiotensin-converting enzyme. Liver production of AGT is regulated mainly at the transcriptional level.5–6 Sequence alterations at position −6 and −20 have been shown to affect transcriptional efficiency of the promoter.7–11 There is a positive correlation between plasma AGT concentration, RAS activity, and blood pressure.5,6,12–13 The AGT gene has been implicated in hypertension by association and linkage studies.13–15

Previously, we described 5 haplotypes (A wild-type to E) at the AGT promoter created by nucleotide substitutions at positions −6, −20, −152, and −217 relative to the transcription start (Figure 1).16 Homozygosity for the B-haplotype (−6:A, −20:C, −152:G, −217:C) conferred an 8-fold increased risk for cSVD in our cohort. The association was independent of hypertension, suggesting that the effect of the haplotype is mediated through the local RAS of the brain rather than through the systemic RAS.

The B-haplotype differs from the wild-type A at positions −6 (G→A) and at −20 (A→C). We hypothesize that either one of these single nucleotide polymorphisms (SNPs) or their combination alters promoter activity of the AGT gene in the brain.
which may then promote development of cSVD through increased cerebral RAS activity. To test this hypothesis, we investigated AGT promoter activity dependent on the promoter haplotypes and on the single $−6A$ and $−20C$ substitutions in astrocytes, which represent the major source of cerebral AGT, and in hepatocytes, which represent the major source of systemic AGT.\(^5\)

Because it is also possible that the association between the B-haplotype and cSVD is not functional but is rather caused by linkage disequilibrium (LD), we genotyped 5 additional sequence variations scattered over the AGT gene and estimated the magnitude of LD between the new SNPs and the promoter haplotypes. Based on these data, we performed a cladistic analysis and investigated the evolutionary relatedness of the haplotypes.

### Materials and Methods

#### Cell Culture

A172 cells were maintained in DMEM containing 10% fetal bovine serum, 4.5 g/L glucose, 100 000 U/L penicillin, and 0.1 g/L streptomycin, and HepG2 cells were maintained in DMEM containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, 100 000 U/L penicillin, and 0.1 g/L streptomycin.

#### Construction of Plasmids

Genomic DNA from subjects homozygous for the A-, B-, C-, D-, or E-haplotypes was amplified by polymerase chain reaction (PCR) between positions $−1222$ and $+44$ (AF424741) and cloned into XhoI/HindIII sites of the pGL3-basic reporter vector (Promega) upstream of the firefly luciferase gene.

The reporter vector including the A-haplotype was used to introduce the $−6A$ (MutAB-6) and the $−20C$ substitutions (MutAB-20) by oligonucleotide-directed mutagenesis. To introduce the $−6A$ mutation, a fragment spanning positions $−120$ to $+10$ (primers: 5\'-GATCCCCAGCTGTTGCTGCGCAAATGA-3\' and 5\'-AGCA-GCTTCTCCCTTGCCC-3\') and a fragment spanning positions $−53$ to $+44$ (primers 5\'-GCTCCATCCCAACCCCTCA-3' and 5\'-ATTGAAAGCCCTGGTTACCTTCTGCTTAG-3') were created. The 2 fragments were then combined by amplification with the primers 5\'-GATCCGAGCTGTTGCTGCGCAAATGGA-3' and 5\'-ATTGAAAGCCCTGGTTACCTTCTGCTTAG-3'). The $−20C$ mutation was introduced similarly except that a fragment spanning position $−120$ to $−14$ was created in the first step (primers 5\'-GATCCGAGCTGTTGCTGCGCAAATGGA-3' and 5\'-TCACTGAGGCGGCTATTAGATGTCAG-3'). The combined fragments were subcloned into the PjM/HindIII sites of the plasmid carrying the A haplotype. All inserts were verified by DNA sequencing.

#### Transient Transfection Assays

Cells were plated at a density of $4 \times 10^5$ cells/60-mm dish and transfected 24 hours later by calcium phosphate coprecipitation with the reporter constructs (10 µg). In each experiment, phRG-TK control plasmid (20 ng) was cotransfected to normalize for transfection efficiency. Luciferase activity was measured 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega) on a LUMAT LB9501 luminometer (Berthold). All measurements were performed in triplicate and have been repeated in at least 3 independent experiments. Results are expressed as the ratio of firefly to renilla luciferase activity (±SEM). Statistical significance was tested by the Student t test and 1-way ANOVA (SPSS for Windows 10.0.5.0).

#### Subjects and Design

The study population consisted of participants of the Austrian Stroke Prevention Study, a single-center, prospective, follow-up study on the cerebral effects of vascular risk factors in the normal elderly population of Graz, Austria. Study design, sampling procedure, and definition of vascular risk factors have been previously described.\(^6,7\) The present study consisted of those 662 individuals who underwent both magnetic resonance imaging analyses and DNA sampling. There were 357 women and 305 men. The mean age was 63.1 years. The sample consisted exclusively of whites of central European origin. The study was approved by the ethical committee of the University of Graz. All subjects gave informed consent to participate in the study.

#### Mutation Screening and Genotyping

We screened the promoter region upstream of the haplotypes (positions $−704$ to $−245b$) and the 3' UTR (positions $+11407$ to $+11712b$) for SNPs. Genomic DNA was PCR-amplified using the oligonucleotides: fragment 1 (spanning position $−532$): 5\'-GC-clamp-GGTCAGTTGAACTTACATTCCGGTGC-3' and 5\'-CAAAAGAGGCTT-AACCAATGCAAAAG; fragment 2 (spanning position $−386$): 5\'-GG-clamp-AAGTTTGCGAAGTGCGGGCAAG and 5\'-GCTTAG-AAGTCAACATCTACGACG; and fragment 3 (spanning positions $+11407$ and $+11712b$): 5\'-GC-clamp-ACCTAAAACACTTC- AAAGGACTGTC-3' and 5\'-CATTGCTTCTGTTGTGTATATTAG-3'. The sequence of the GC-clamp was 5\'-CAGCCCGCCGGCCGCCGCCC- CGCGCCGCCGGCCGCCC-3'. PCR products were screened for the presence of mutations by TTGE (Dcode Universal mutation detection system; Bio-Rad Laboratories). Melting domain map was calculated with the MacMelt algorithm (Bio-Rad Laboratories). Heterozygous DNA samples were used as positive controls on each gel to check gel resolution efficiency. At least 3 samples with a distinct banding pattern on TTGE were sequenced (CEQ 2000XL DNA analyses system; Beckman Coulter). The M2357 polymorphism was genotyped using PCR-refraction fragment length polymorphism.\(^8\)

#### Statistical Analysis

The haplotype tree was inferred with the maximum likelihood method, using DNAML 3.573c (Phylip; http://evolution.genetics.
Transcriptional Activities of the MutAB-6 and the MutAB-20 Constructs

To elucidate the individual effect of the \(-6\):A and \(-20\):C substitutions distinguishing the B-haplotype from the A-haplotype, we transiently transfected A172 and HepG2 cells with the reporter constructs containing either only the \(-6\):A substitution (MutAB-6) or the \(-20\):C substitution (MutAB-20) (Figure 2A). The promoter activities of these constructs were compared with the promoter activities of both the A- and B-haplotypes and of MutAB-6 and MutAB-20 constructs in hepatocytes. MutAB-6 had a significantly increased promoter activity (1.6±0.07-fold, \(P<0.001\)) compared with the A-haplotype, but its transcriptional activity was still significantly lower than that of the B-haplotype (\(P<0.001\)). MutAB-20 had similar promoter activity in astrocytes (\(P=0.3\)) as the A-haplotype. By contrast, in hepatocytes promoter activity of MutAB-6 was similar to that of the A-haplotype (\(P=0.2\)), whereas MutAB-20 had a 2.5±0.06-fold increased promoter activity (\(P<0.001\)) (Figure 2B). This is comparable with the effect of the B-haplotype in these cells (\(P=0.8\)).

Pairwise LD and Haplotype Analyses

We genotyped our cohort for the SNPs \(-532\):C\(\rightarrow\)T and \(-386\):G\(\rightarrow\)A in the promoter region, and for the SNPs \(+11535\):C\(\rightarrow\)A and \(+11535\)::delC in the 3’-UTR as well as for the M235T polymorphism. Allele and genotype proportions for all the SNPs were in Hardy–Weinberg equilibrium (Table 1). The allele frequencies in our cohort were similar to the previously published frequencies in a sample of white subjects (Utah CEPH pedigrees) (Table 1).

The magnitude of LD among the SNPs is shown in Figure 3. There was no decline of LD with physical distance (constant LD between the SNPs as assessed by \(D^2\) versus exponential decline models, \(P>0.1\)). Average LD between the SNPs as assessed by \(D^2\) was 0.841.

Including the 5 newly investigated SNPs and the 4 SNPs determining the promoter haplotypes, we inferred 9 haplotypes over the AGT gene in our cohort. These haplotypes coded from H1 to H9 based on their frequencies are described in Table 2. The evolutionary relatedness of these haplotypes in form of a haplotype tree is presented in Figure 4. Overall, 80% of the promoter B-haplotype was present on H4, 12% on H8, and 8% on H9, both closely related to H4. The H4 AGT haplotype is equivalent to the published haplotype (\(P<0.001\)) (Figure 3). This is comparable with the effect of the B-haplotype in these cells (\(P=0.8\)).
chimpanzee sequence (AF193457.1), suggesting that the B-haplotype represents an ancient promoter variant.

Discussion
In the current study, we show that the AGT promoter B-haplotype, which was associated with cSVD in our previous investigation, significantly enhances basal transcriptional activity of the AGT gene. The association was independent of hypertension, indicating that it might be mediated through an altered cerebral AGT expression. We found that the B-haplotype increases promoter activity more strongly in astrocytes, which are the major source of cerebral AGT, than in hepatocytes, which are the major source of systemic AGT.

If tissue RAS is regulated at the AGT level as is the systemic RAS, then a higher AGT transcription in astrocytes may lead to elevated AT-II concentration in the brain. AT-II is a potent regulator of vascular tone, it promotes vascular smooth muscle cell growth, and modulates the production of extracellular matrix proteins. It enhances the activity of NADH/NADPH oxidase and of extracellular superoxide dismutase in the vessel wall. Alteration in the local availability of AT-II may therefore result in an imbalance of physiological processes such as brain perfusion, autoregulation of cerebral blood flow, the oxidative state of the vessel wall, or function of the blood–brain barrier, and by these mechanisms may lead to the development of cSVD.

The B-haplotype contains 2 nucleotide substitutions, −6:G→A and −20:A→C, compared with the wild-type A-haplotype. We found that in contrast to hepatocytes, the effect of the B-haplotype in astrocytes is dependent on the presence of both of these substitutions, because neither the −6:A nor the −20:C mutation alone was able to enhance promoter activity to the level observed for the B-haplotype. This is in line with our previous genetic epidemiological data, showing that the B-haplotype is stronger and more significantly associated with
cSVD than either the −20:C or the −6:A alleles. An interaction between the −6:A and the −20:C mutation is biologically plausible because these 2 mutations are located in 2 distinct regulatory elements of the core promoter.9,11

The 5 promoter haplotypes showed a cell type-specific and differential effect on the transcriptional activity, suggesting that the effect of the −20:C and the −6:A mutations are further modulated by other sequence variations at the AGT promoter. This is in line with previous findings indicating that the sequence between positions −25 to −1 mediate the responsiveness of the core promoter to upstream cis-acting elements.8

A further, although indirect, support for the functional relevance of the B-haplotype is suggested by its ancient origin. It had been hypothesized that the AGT haplotype carrying the −6:A and the 235T mutations was advantageous during human evolution in the low-salt environment of Africa by allowing for a higher salt reabsorption through enhanced AGT expression and aldosterone secretion.13,24 When our ancestors moved to the high-salt environment in Europe, however, this ancient haplotype may have become disadvantageous. The B-haplotype is in complete LD with the 235T variant, and the enhanced risk for cSVD associated with 235T variant might be caused by LD with the B-haplotype.5,3 Importantly, although the B-haplotype contains the −6:A substitution and also perfectly predicts the presence of C at position 4072, which codes for the 235T variant, only 34% of the 235T allele is associated with the B-haplotype and may therefore enhance the risk for cSVD.

A strength of our study is that we investigated the effect of naturally occurring promoter haplotypes on the transcriptional activity of the AGT gene, and that we dissected the individual effect of the −6:A and −20:C mutations present on the B-haplotype. We used astrocytes as a cellular model for cerebral AGT expression. It is important to realize, however, that other cell types, like vascular smooth muscle cells, endothelial cells, or eventually neurons, may also contribute to AGT production at the site of cSVD.

In conclusion, we showed that the combination of the −6:A and −20:C mutations in the B-haplotype enhances angiotensinogen promoter activity in astrocyte. This finding together with our previous epidemiological data suggests that increased cerebral RAS activity is involved in the cause of cSVD. Further studies are needed to investigate the role of the B-haplotype in independent populations, and to elucidate the biological role of the SNPs at the AGT gene in cSVD. A causal relation between B-haplotype and cSVD independent of hypertension points to intervention in the RAS as a possible treatment strategy in cSVD, a highly prevalent entity in elderly subjects for which no treatment has been proven to be effective until now.

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