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Genetic variants of the *NOTCH3* gene in the elderly and magnetic resonance imaging correlates of age-related cerebral small vessel disease

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Cerebral small vessel disease-related brain lesions such as white matter lesions and lacunes are common findings of magnetic resonance imaging in the elderly. These lesions are thought to be major contributors to disability in old age, and risk factors that include age and hypertension have been established. The radiological, histopathologic and clinical phenotypes of age-related cerebral small vessel disease remarkably resemble autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy, which is caused by mutations in *NOTCH3*. We hypothesized that genetic variations in *NOTCH3* also play a role in age-related cerebral small vessel disease. We directly sequenced all 33 exons, the promoter and 3′-untranslated region of *NOTCH3* in 195 participants with either coalescent white matter lesions or lacunes and compared the results to 82 randomly selected participants with no focal changes on magnetic resonance images in the Austrian Stroke Prevention Study. We detected nine common and 33 rare single nucleotide polymorphisms, of which 20 were novel. All common single nucleotide polymorphisms were genotyped in the entire cohort (*n* = 888), and four of them, rs1043994, rs10404382, rs10423702 and rs1043997, were

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associated significantly with both the presence and progression of white matter lesions. The association was confined to hypertensives, a result which we replicated in the Cohorts for Heart and Ageing Research in Genomic Epidemiology Consortium on an independent sample of 4773 stroke-free hypertensive elderly individuals of European descent ($P = 0.04$). The 33 rare single nucleotide polymorphisms were scattered over the *NOTCH3* gene with three being located in the promoter region, 24 in exons (18 non-synonymous), three in introns and three in the 3′-untranslated region. None of the single nucleotide polymorphisms affected a cysteine residue. Sorting Intolerant From Tolerant, PolyPhen2 analyses and protein structure simulation consistently predicted six of the non-synonymous single nucleotide polymorphisms (H170R, P496L, V1183M, L1518M, D1823N and V1952M) to be functional, with four being exclusively or mainly detected in subjects with severe white matter lesions. In four individuals with rare non-synonymous single nucleotide polymorphisms, we noted anterior temporal lobe hyperintensity, hyperintensity in the external capsule, lacunar infarcts or subcortical lacunar lesions. None of the observed abnormalities were specific to cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy. This is the first comprehensive study investigating (i) the frequency of *NOTCH3* variations in community-dwelling elderly and (ii) their effect on cerebral small vessel disease related magnetic resonance imaging phenotypes. We show that the *NOTCH3* gene is highly variable with both common and rare single nucleotide polymorphisms spreading across the gene, and that common variants at the *NOTCH3* gene increase the risk of age-related white matter lesions in hypertensives. Additional investigations are required to explore the biological mechanisms underlying the observed association.

Keywords: *NOTCH3*; cerebral small vessel disease; genetics; MRI; ageing

Abbreviations: AGES = Ageing Gene–Environment Susceptibility-Reykjavik Study; ARIC = Atherosclerosis Risk in Communities Study; CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy; CHARGE = cohorts for heart and ageing research in genomic epidemiology consortium; NCBI = National Centre for Biotechnology Information; SIFT = Sorting Intolerant From Tolerant; SNP = single nucleotide polymorphism

Introduction

Correlates of age-related cerebral small vessel disease, such as white matter lesions and lacunes are common (Launer *et al.*, 2006), can progress rapidly (Schmidt *et al.*, 2007) and are associated with cognitive impairment (Breteler *et al.*, 1994; Longstreth *et al.*, 2005) to dementia (O'Brien *et al.*, 2003; Vermeer *et al.*, 2003*b*), gait disturbances and falls (Rosano *et al.*, 2006; Srikanth *et al.*, 2009). They are also predictors of future disability (Inzitari *et al.*, 2009). Besides age and arterial hypertension, causative factors are widely unknown (O'Brian *et al.*, 2003). The estimated heritability index of white matter lesions is in the range of 55–73% (Carmelli *et al*. 1998; Atwood *et al.*, 2004), which is a clear indication that genetic factors play a major aetiological role. Age-related cerebral small vessel disease shows remarkable radiological and histopathological similarities with cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL), a monogenic form of systemic non-amyloid arteriopathy primarily affecting the brain (Adib-Samii et al., 2010; Chabriat *et al.*, 1995, 2009; Joutel *et al.*, 1996; Dichgans *et al.*, 1998; Desmond *et al.*, 1999; Reyes *et al.*, 2009). CADASIL is caused by mutations in the *NOTCH3* gene (Joutel *et al.*, 1996). Notch3 plays a key role in the functional and structural integrity of small arteries by regulating vascular smooth muscle cell differentiation, maturation and phenotypic behaviour (Artavanis-Tsakonas *et al*., 1999; Domenga *et al.*, 2004; Wang *et al.*, 2008). We hypothesized that Notch3 not only plays a role in CADASIL but is also involved in the development of age-related cerebral small vessel disease, with both common and rare single nucleotide polymorphisms (SNPs) of the *NOTCH3* gene contributing to the risk of this multi-factorial disorder. We took a two step approach to evaluate this hypothesis in the setting of the Austrian Stroke Prevention Study. First, we screened the promoter, the coding and the 3′ untranslated region of the *NOTCH3* gene for the presence of mutations in all individuals with either coalescent white matter lesions or lacunes and compared the frequency of genetic variants to a random subset of participants from the Austrian Stroke Prevention Study who were free from focal lesions on MRI. We then genotyped the whole cohort ($n = 888$) for the observed common SNPs (minor allele frequency $\geqslant 5\%$) and assessed their association with the presence of white matter lesions and lacunes as a primary phenotype and white matter lesion progression as a secondary phenotype. We sought to replicate our finding in an independent sample of 8545 stroke-free individuals of European descent in the Cohorts for Heart and Ageing Research in Genomic Epidemiology (CHARGE) Consortium including six community-based cohorts with brain MRI scans and genome-wide genotype data (Fornage *et al.*, 2011). We further elucidated the effects of the rare (minor allele frequency $<5\%$) non-synonymous SNPs in the *NOTCH3* gene by studying their association with CADASIL-specific clinical and MRI characteristics and by analysing the possible functionality of non-synonymous SNPs using bioinformatical tools.

Materials and methods

Study population

The Austrian Stroke Prevention Study is a prospective cohort study on the effects of vascular risk factors on brain structure and function in cognitively normal middle-aged and elderly

inhabitants of Graz, Austria; details have previously been described (Schmidt *et al.*, 1994, 1999). Briefly, participants ($n = 2007$) were selected randomly from the official community register and all participants were free from stroke and dementia. The study protocol was approved by the responsible ethics committee. In an initial study panel that took place between 1991 and 1994, subjects ($n = 509$) randomly selected from the entire cohort underwent an extended diagnostic work-up including brain MRI and cognitive testing. To enlarge the cohort with imaging and neuropsychological assessments, an additional 567 individuals were selected randomly in a second panel between 1999 and 2003. They underwent identical imaging and cognitive test procedures. Participants of the first and second panels were pooled, which resulted in a total of 1076 individuals with brain MRI and neuropsychological evaluation. Clinical history, blood tests, MRI and cognitive assessments were always performed on the same day. Historic information and laboratory findings at baseline were considered for risk factor diagnosis. The definitions have previously been described (Schmidt *et al.*, 1999). A history of migraine was taken in all study participants and all individuals underwent dementia screening by the Mini-Mental State Examination (Folstein *et al.*, 1975). For assessment of depressive mood, we applied the 'Eigenschaftswörterliste', a validated multi-dimensional tool consisting of a list of given adjectives describing the emotional state of a person (Janke, 1978). In the present study, 888 participants of the Austrian Stroke Prevention Study with brain MRI and DNA samples were included. Their mean age was 65.2 ± 8.0 years. There were 505 (56.9%) women. Mean systolic blood pressure from three measurements taken during clinical examination was 143.8 ± 23.16 mmHg (range 85–220), mean diastolic blood pressure was 87.7 ± 10.32 mmHg (range 60–123). Hypertensives were defined as having a mean systolic blood pressure $\geqslant 140$ mmHg or a diastolic blood pressure $\geqslant 90$ mmHg or being on anti hypertensive therapy. A total of 641 (72.2%) participants were hypertensive and 251 (23.3%) were on antihypertensive therapy. In total, 86 (9.7%) had diabetes and 304 (34.2%) had cardiac disease based on clinical, ECG and echocardiography results. Altogether, 152 (17.1%) subjects reported migraine and 91 (10.2%) reported depressed mood. The mean Mini-Mental State Examination score of the sample was 27.4 ± 1.7. At least one follow-up examination at 3 and 6 years with identical protocols was carried out in 534 subjects. There were 500 and 300 subjects with 3- and 6-year follow-up MRI scans, respectively.

Magnetic resonance imaging

All scans were obtained on a 1.5 Tesla scanner (Philips Medical Systems) with protocols described previously (Schmidt *et al.*, 1999). White matter lesion grade, volume and lacunes were determined in each study participant at baseline and follow-ups. White matter lesions were assessed by two experienced investigators (R.S. and C.E.). There were 717 (80.7%) participants with white matter lesions of any grade. In total, 195 (21.9%) subjects had early confluent or confluent lesions. The methods used for volume measurements have been described elsewhere (Schmidt *et al.*, 1999, 2005). Mean white matter lesion load was 3.04 cm^3 (range 0–78) at baseline investigation. The annual change of white matter lesion volume was calculated by dividing the volume change by years of follow-up. The mean annual change in white matter lesion load observed in our study participants was 0.22 cm^3 (range 0–4.99). Lacunes were defined as focal lesions that involved the basal ganglia, internal capsule, thalamus or brainstem, not exceeding a maximum diameter of 15 mm and occurred in 80 (9.2%) subjects. Incident lacunes at any follow-up were determined by direct comparison of baseline and follow-up scans. Incident lacunes were seen in 10 (2.0%) study participants. In carriers of rare non-synonymous SNPs, MRI images were also reviewed for findings suggestive of CADASIL. These included anterior temporal lobe hyperintensity, hyperintensity of the external capsule and subcortical lacunar lesions (Markus *et al.*, 2002; van den Boom *et al.*, 2003). As in subjects with common SNPs, we also recorded lacunes, white matter lesion volume at baseline and annual progression of lesions in this subset of participants. Microbleeds could not systematically be assessed as only a small portion of participants in the Austrian Stroke Prevention Study underwent MRI with gradient echo sequences.

DNA analyses

DNA sampling

Genomic DNA was extracted from ethylenediaminetetraacetic acid–blood using the standard phenol/chloroform method. A portion of the genomic DNA was amplified by REPLI-g® Screening Kit (QIAGEN) and was used as template for the mutation screening. The original genomic DNA was used for confirmatory sequencing of detected SNPs and for genotyping the common SNPs using the TaqMan® assay.

Mutation screening

The promoter, all 33 exons (including the intronic flanking regions) and 3′-untranslated regions were amplified by PCR in separate reactions. Exon 24 was subdivided into two overlapping PCRs (24–1, 24–2) and exon 33 into 6 overlapping PCRs (33–1, 33–6). The PCR products were then purified with Wizard® SV Gel and PCR Clean-up kit as prescribed by Promega, verified by gel electrophoresis and sequenced using BigDye® Terminator v1.1 Cycle Sequencing (Applied Biosystems) on a 3730 DNA Analyser (Applied Biosystems/HITACHI). All reagents, PCR primers and conditions are given in the Supplementary material. Electropherograms were analysed with the SeqScape v2.5 (Applied Biosystems) and the Sequencing Analysis 5.2 (Applied Biosystems) algorithms. The messenger RNA sequence of the National Centre for Biotechnology Information (NCBI; accession number NM_000435, version NM_000435.2) was used as the reference sequence and for numbering of mutations at the DNA level. The corresponding amino acid sequence originated from GenBank (accession number U97669, version U97669.1), positions of the mutations are given related to the ATG translation start site at the protein level. For the final annotation of detected common SNPs, The Single Nucleotide Polymorphism database of the NCBI was used (www.ncbi.nlm.nih.gov/SNP/). All samples showing a sequence alteration during the screening procedure were resequenced

using genomic DNA as a template in both 3′ and 5′ directions in order to define mutations with high confidence.

Genotyping of common single nucleotide polymorphisms

Real-time PCR with sequence-specific TaqMan® probes (Ingenetix; Vienna, Austria) were used for genotyping common SNPs (minor allele frequency $\geqslant$5%; rs11882186, rs1043994, rs61749020, rs10423702, rs1043996, rs1043997, rs1044006, rs4809030 and rs1044009) in 888 participants of the Austrian Stroke Prevention Study cohort for whom both MRI and DNA samples were available. TaqMan® assays were analysed on a 7900HT Fast Real-time PCR system (Applied Biosystems). Detailed genotyping conditions are given in the Supplementary material. One common SNP at the *NOTCH3* gene, rs10404382, was genotyped earlier on the Illumina Human610-Quad BeadChip and was also included in the analyses.

Functional assessment of rare single nucleotide polymorphisms

Sorting Intolerant From Tolerant and PolyPhen2

The Sorting Intolerant From Tolerant (SIFT) algorithm (J. Craig Venter Institute, version 4.0.3) was used to predict whether rare SNPs affect the Notch3 protein function. SIFT uses sequence homology for calculating whether an amino acid substitution at a given position is tolerated or affects the protein function. Substitutions are more tolerated at positions with low conservation and more intolerant with high conservation. In order to get related homologous sequences for the input query sequence, a Basic Local Alignment Search Tool search was performed using the NCBI non-redundant database. Only related sequences with a maximum identity percentage of 90 were used for this analysis. SIFT is available at http://sift.jcvi.org.

PolyPhen2, a revised version of PolyPhen, allows prediction of the impact of missense mutations on the function of encoded proteins by performing various sequence and structure analyses, yielding a probability of altered function. We have used the data set HumDiv to test and train the predictions made by PolyPhen2. Predictions are characterized as either benign, possibly damaging or probably damaging on the output. PolyPhen2 is available at http://genetics.bwh.harvard.edu/pph2.

Protein simulation by homology modelling and threading

The 3D structure of Notch3 was not available in the Protein Data Bank database (Sussman, 1999). Therefore, parts of the structure have been determined by homology modelling and threading. Homology modelling is a knowledge-based structure prediction where a protein sequence with an unknown structure (the target) is aligned with one or more protein sequences with known structures (the templates). By the threading method, the sequence with an unknown structure is fitted into a variety of existing structures. The model building can be done by using profile–profile matching and secondary structure. The homology modelling was done with SWISS-MODEL and the threading with Phyre (Protein HomologY Recognition Engine), a successor of 3D-PSSM (Kelley and Sternberg, 2009). To study putative structural effects of the mutations, the affected residues inside the domains of Notch3 were interchanged and an energy minimization was performed. These replacements were performed on the Swiss-Pdb viewer (Guex and Peitsch, 1997). The structural changes due to the mutations are visualized and the energy of the molecular force field (GROMOS96 force field) calculated (Li *et al.*, 2008).

Statistical analyses

Genotypes were coded according to the minor alleles as 0 for homozygous for the major allele, 1 for heterozygous and 2 for homozygous for the minor allele. All common SNPs were tested for Hardy Weinberg equilibrium. The linkage disequilibrium plot was drawn using common SNPs genotyped in the Austrian Stroke Prevention Study cohort by HaploView 4.2 (Barrett *et al.*, 2005) using the standard (D′/LOD) linkage disequilibrium colour scheme. Linkage disequilibrium values are given by R^2. The haplotype blocks were estimated using the 'solid spine of LD' method, which defines haplotype blocks by the first and the last SNPs being in strong linkage disequilibrium with all intermediate markers. Linkage disequilibrium does not necessarily appear between intermediate SNPs. Linear trend test with 1 degree of freedom was used to assess the effect of genotypes on the presence of white matter lesions and lacunes assuming additive genetic effects. Logistic regression analyses were used to assess the association between the common SNPs and the presence of white matter lesions as well as lacunes upon adjustment for age (Model 1) and age, sex, hypertension, cardiac disease and diabetes (Model 2). Multiple linear regressions were used to assess the effect of common SNP genotypes on the annual change of white matter lesion volume, as measure of white matter lesions progression upon adjustment for age (Model 1) and age, sex, hypertension, cardiac disease and diabetes (Model 2). Both regression analyses were also conducted with stratifications for hypertension (yes/no).

Replication

The replication sample comprised 8545 individuals of European descent (aged 69.5 years; 42.6% men) belonging to six community-based cohort studies including the Ageing Gene–Environment Susceptibility-Reykjavik Study (AGES); the Atherosclerosis Risk in Communities (ARIC) Study; the Cardiovascular Health Study; the Framingham Heart Study; and two cohorts from the Rotterdam Study. All studies collaborated within the CHARGE consortium neurology working group. Details of the goals and organization of the CHARGE consortium have previously been described (Psaty *et al.*, 2009). A total of 4773 hypertensive (AGES: 1959, ARIC: 157, Cardiovascular Health Study: 1082, Framingham Heart Study: 940, Rotterdam Study I: 269, Rotterdam Study II: 366) and 3772 normotensive (AGES: 508, ARIC: 633, Cardiovascular Health Study: 1102, Framingham Heart Study: 1218, Rotterdam Study I: 111, Rotterdam Study II: 200) individuals were included in the present analyses. Details of cohort selection, risk factor assessment and white matter lesion assessment in each study, as well as genotyping procedures, have been described (Fornage *et al.*, 2011) and are given in more detail in the Supplementary material. Briefly, each study performed

genome wide genotyping on different platforms and after extensive quality control all studies used their genotype data to impute to the 2.5 million non-monomorphic, autosomal SNPs described in the HapMap's CEU (Utah residents with Northern and Western European ancestry from the Centre de'Etude du Polymorphism Humain collection) panel. The MRI scan protocol to detect white matter lesions either used a fluid attenuation inversion recovery sequence or proton density sequences. In participants from the AGES-Reykjavik, Framingham Heart Study and Rotterdam Study, white matter lesion volume was estimated on a quantitative scale using custom written computer programmes based on an automatic segmentation algorithm or a semi-automatic segmentation analysis, in participants of the ARIC and Cardiovascular Health Studies, white matter lesion volume was estimated on a semi-quantitative scale. Within each study, a linear regression model was used to evaluate the association of the SNP with the natural log-transformed volume of white matter lesions. The CHARGE consortium used the term white matter hyperintensity as a synonym for white matter lesions. An additive genetic model was assumed and significance of association was estimated using a 1 degree of freedom trend test relating genotype dosage, zero to two copies of the minor allele, to the phenotype. Analyses were adjusted for age, sex and total intra-cranial volume (except in the ARIC and Cardiovascular Health Studies, which inherently adjust for intracranial volume). In addition, the ARIC and Cardiovascular Health Studies adjusted for study site, and the Framingham Heart Study adjusted for familial structure. Within each study, analyses were performed separately in normo- and hypertensives. Fixed-effect meta-analyses with inverse variance weighting and forest plots were done according to hypertension status using the Comprehensive Meta Analysis Version 2.2 software (Borenstein *et al.*, 2009). For estimating heterogeneity, Cochran's Q and I^2 statistics were calculated.

Results

NOTCH3 sequence variations

We sequenced the *NOTCH3* gene including the promoter, all 33 exons with the intronic flanking regions and the 3′-untranslated region in all 195 participants of the Austrian Stroke Prevention Study with early confluent or confluent white matter lesions or lacunes and in 82 randomly selected participants with normal MRI. We found 42 SNPs scattered over the gene, with four located in the promoter region, 30 in exons (19 non-synonymous, 11 synonymous), five within introns and three in the 3′-untranslated region. None of the SNPs affected a cysteine residue. In 13 exons, we did not detect mutations. According to the functional domains of the Notch3 protein, 18 SNPs (11 non-synonymous, seven synonymous) were in the epidermal growth factor-like repeats, two SNPs (one non-synonymous, one synonymous) in the Lin12/Notch repeat domain, three (two non-synonymous, one synonymous) in the Notch domain, one non-synonymous SNP in the transmembrane domain, three SNPs (two non-synonymous, one synonymous) in the ankyrin repeats domain and three (two non-synonymous, one synonymous) in the low complexity region domain. The distribution of SNPs over the *NOTCH3* gene and protein is shown in Fig. 1.

Common single nucleotide polymorphisms and magnetic resonance imaging findings

Among the 42 SNPs, nine had a minor allele frequency minor allele frequency $\geqslant 5\%$ in the subset of individuals with normal MRI. All of these common SNPs were described in the Single Nucleotide Polymorphism database and their characteristics are given in Supplementary Table 1. All common SNPs, except for rs1044009, which was excluded from further analyses, were in Hardy Weinberg equilibrium. Linkage disequilibrium among the SNPs in our cohort is shown in Fig. 2. We investigated the association between common SNPs and the presence of white matter lesions and lacunes by assuming an additive genetic effect for the minor alleles at each SNP (Supplementary Table 2). Carriers of the minor allele at SNPs rs1043994, rs10404382 and at rs10423702 more commonly had white matter lesions. White matter lesions were seen in 532 (79.2%) AA, 160 (85.1%) AG and 16 (94.1%) GG genotype carriers, at rs1043994 ($P = 0.022$); in 488 (80.5%) AA, 155 (87.1%) AC and 13 (92.9%) CC genotype carriers at rs10404382 ($P = 0.022$); and in 535 (79%) individuals with the

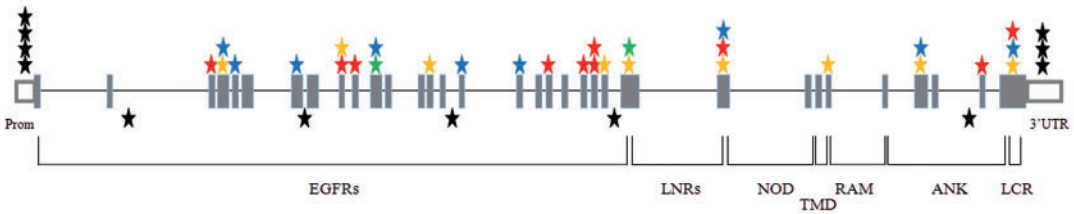

Figure 1 Distribution of detected single nucleotide polymorphisms over the promoter region, exons and 3′-untranslated region of the *NOTCH3* gene. Corresponding protein domains shown below. Functional annotation of mutations is colour coded: red star = non-synonymous mutation; blue star = synonymous mutations; orange star = non-synonymous mutation occurring only in participants with white matter lesions; green star = synonymous mutation occurred only in participants with white matter lesions; black star = mutation in promoter region, 3′ untranslated region and introns, respectively. ANK = ankyrin repeats; EGFR = epidermal growth factor-like repeat; LCR = low complexity region; LNR = Lin12/Notch repeat; NOD = Notch domain; Prom = promoter; RAM = RBP-Jκ-associated molecule domain; TMD = transmembrane domain; 3′-UTR = 3′-untranslated region.

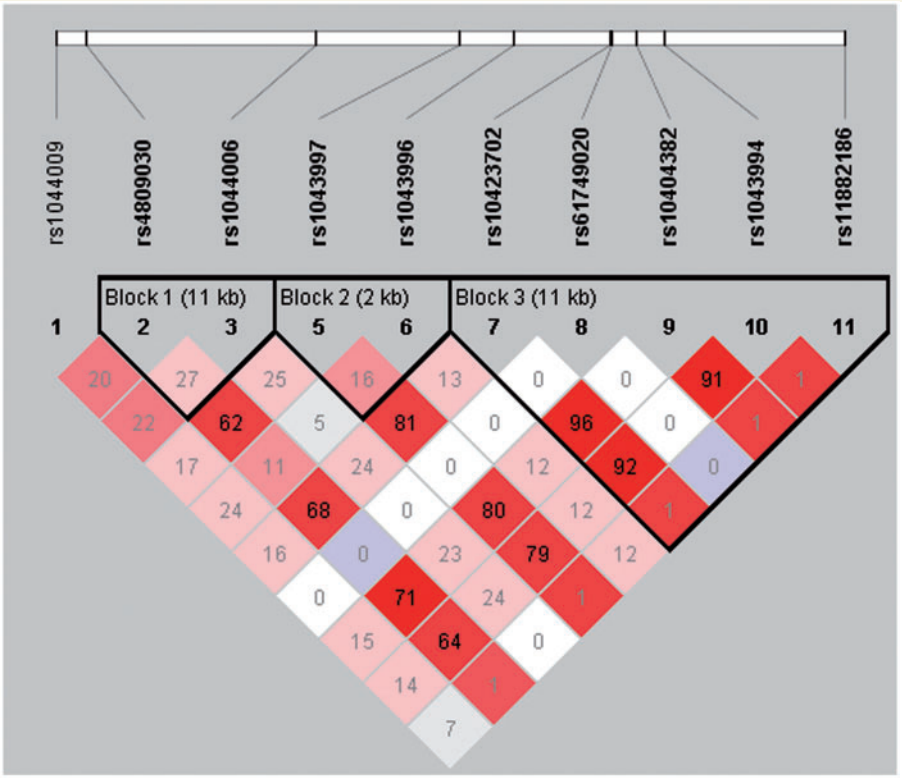

Figure 2 Linkage disequilibrium plot of common single nucleotide polymorphisms together with inferred haplotype blocks. (*Top*) Distribution of common SNPs on chromosome 19 over the *NOTCH3* gene. The linkage disequilibrium values are *R*-squared with standard (*D′/LOD*) colour scheme. Each black triangle depicts haplotype blocks.

AA, 163 (85.8%) with the AG and 14 (93.3%) with the GG genotype at rs10423702 ($P = 0.015$). Logistic regression analysis with adjustment for age (Model 1) and adjustment for age, gender, hypertension, diabetes and cardiac disease (Model 2) revealed four of the common SNPs—rs1043994, s10404382, rs10423702 and rs1043997—to be significantly related to the presence of white matter lesions (Table 2). Stratification of the analysis by the presence of hypertension showed that the effect of the four SNPs was only present in hypertensives but not in normotensives. As shown in Table 2, odds ratios were in the range 2.1–3.4 for the presence of one minor allele at these SNPs in hypertensives ($P < 0.01$), while there was no significant association in normotensives. The frequency of white matter lesions over the genotypes upon stratification is given in the Supplementary material. As can be seen in Table 3, the SNPs were not only associated with the presence, but also with the progression of white matter lesions. The effect was strongest in subjects with hypertension. Importantly, the size and strength of the association changed only marginally when additional adjustment for baseline white matter lesions load was done (rs1043994: $\beta = 0.125$; $P = 0.07$; rs10404382: $\beta = 0.142$; $P = 0.05$; rs10423702: $\beta = 0.141$; $P = 0.04$; rs1043997: $\beta = 0.087$; $P = 0.2$). None of the SNPs showed associations with the presence of lacunes (rs1043994: odds ratio = 1.05, $P = 0.89$; rs10404382: odds ratio = 1.21, $P = 0.44$; rs10423702: odds ratio = 1.03, $P = 0.89$; rs1043997: odds ratio = 1.16, $P = 0.54$).

Replication study

We sought to replicate our results on the SNP rs10404382, which was associated with the highest risk for white matter lesions in the Austrian Stroke Prevention Study cohort, within the CHARGE consortium including the AGES, ARIC, Cardiovascular Health Study, Framlingham Heart Study and Rotterdam Studies I and II. There were 4773 hypertensive (AGES: 1959, ARIC: 157, Cardiovascular Health Study: 1082, Framlingham Heart Study: 940, Rotterdam Study I: 269, Rotterdam Study II: 366) and 3772 normotensive (AGES: 508, ARIC: 633, Cardiovascular Health Study: 1102, Framingham Heart Study: 1218, Rotterdam Study I: 111, Rotterdam Study II: 200) stroke-free elderly individuals involved in the replication. Figure 3 displays the results of the meta-analyses in the form of forest plots in these two subgroups as well as the study-specific β-coefficients and 95% confidence intervals (95% CIs). The C allele (allele frequency = 0.12; range = 0.11–0.16) increased white matter lesions burden in hypertensives ($P = 0.04$; $\beta = 0.039$; 95% CI 0.002; 0.076 on the transformed log scale), but not in normotensives ($P = 0.89$; $\beta = 0.002$; 95% CI −0.028 to 0.033). The effect in hypertensives corresponds to an increase of 3.5% of the overall mean white matter lesions burden by one risk allele. There was no evidence of substantial between study heterogeneity in either subgroups (normotensives: Cochran's $Q = 4.183$, $P = 0.53$, $I^2 = 0.00$; hypertensives: Cochran's $Q = 7.59$, $P = 0.18$, $I^2 = 34.15$). At the individual study

Table 1 Association between common single nucleotide polymorphisms and the presence of white matter lesions in the Austrian Stroke Prevention Study cohort

SNP ID	Minor allele	Minor allele frequency	*n*	Odds ratio (95% CIs)	*P*-value
rs11882186[a]	T	12.5	851	1.083 (0.720–1.630)	0.702
rs11882186[b]	T	12.5	851	1.051 (0.694–1.591)	0.814
rs1043994[a]	G	12.7	877	1.677 (1.093–2.572)	**0.018**
rs1043994[b]	G	12.7	877	1.730 (1.121–2.669)	**0.013**
rs10404382[a]	C	12.9	798	1.751 (1.100–2.786)	**0.018**
rs10404382[b]	C	12.9	798	1.801 (1.127–2.879)	**0.014**
rs61749020[a]	C	5.1	857	1.075 (0.606–1.908)	0.804
rs61749020[b]	C	5.1	857	1.099 (0.615–1.964)	0.749
rs10423702[a]	G	12.5	882	1.700 (1.103–2.621)	**0.016**
rs10423702[b]	G	12.5	882	1.727 (1.114–2.676)	**0.015**
rs1043996[a]	C	28.1	795	1.130 (0.835–1.530)	0.428
rs1043996[b]	C	28.1	795	1.154 (0.850–1.567)	0.358
rs1043997[a]	G	13.5	885	1.483 (0.998–2.201)	**0.051**
rs1043997[b]	G	13.5	885	1.510 (1.013–2.249)	**0.043**
rs1044006[a]	A	12.6	833	1.006 (0.690–1.467)	0.976
rs1044006[b]	A	12.6	833	1.033 (0.705–1.514)	0.867
rs4809030[a]	T	10.1	850	1.355 (0.846–2.171)	0.206
rs4809030[b]	T	10.1	850	1.417 (0.879–2.284)	0.152

Odds ratios and *P*-values are given for the effect of the minor alleles, significant SNPs are highlighted in bold.
a Logistic regression analyses Model 1 including age and the corresponding SNP.
b Logistic regression analyses Model 2 including age, sex, hypertension status, diabetes status and the corresponding SNP.
n = number of successfully genotyped individuals; SNP ID = Identification number of SNPs according to the Single Nucleotide Polymorphism database. Available from: http://www.ncbi.nlm.nih.gov/SNP/

level, we saw the strongest and most significant effect in AGES ($P = 0.014$; $\beta = 0.108$; 95% CI 0.022–0.194), and Cardiovascular Health Study ($P = 0.029$; $\beta = 0.62$; 95% CI 0.006–0.118) representing the largest and oldest cohorts.

Clinical characteristics of rare single nucleotide polymorphism carriers

We detected 33 rare SNPs (minor allele frequency <5%) including 20 SNPs, which have not been described previously. As shown in Table 4, three SNPs were located in the promoter region, 24 in exons, three in introns and three in the 3′-untranslated region. Altogether, eight rare SNPs were C to T transitions at CpG dinucleotides. The 24 exonic SNPs included 18 non-synonymous and six synonymous mutations. Fourteen SNPs were located in epidermal growth factor-like repeats, two in Lin12/Notch repeat domains, two in the Notch domain, one in the transmembrane domain, three in the ankyrin repeat domains and two in the low complexity region domain of Notch3. Of these SNPs, 15 were exclusively detected in participants with severe white matter lesions, one in the promoter region, 11 in exons, one in an intron and two in the 3′-untranslated region. Importantly, nine of them were non-synonymous SNPs. Clinical characteristics of the carriers of these nine are shown in Table 5. All were >65 years, and with one exception all had hypertension. In all subjects who underwent repeated MRI scanning, white matter lesions progression was observed. Temporal lobe hyperintensity, hyperintensity in the external capsule, lacunar infarcts or subcortical lacunar lesions were seen in four of the nine individuals. None of these signal changes

Table 2 Association of common single nucleotide polymorphisms with the presence of white matter lesions in normotensive and hypertensive individuals

SNP ID	Normotensives			Hypertensives		
	n	Odds ratio (95% CI)	*P*-value	*n*	Odds ratio (95% CI)	*P*-value
rs1043994	243	1.08 (0.571–2.044)	0.813	634	2.503 (1.342–4.667)	**0.004**
rs10404382	224	0.933 (0.482–1.896)	0.836	573	3.239 (1.559–6.728)	**0.002**
rs10423702	244	1.070 (0.558–2.054)	0.838	637	2.467 (1.324–4.598)	**0.004**
rs1043997	247	0.995 (0.558–1.776)	0.987	637	2.148 (1.204–3.834)	**0.010**

Significant SNPs are highlighted in bold. Odds ratios and *P*-values are given for the presence of one minor allele at the corresponding SNPs after adjustment for age, sex, diabetes status and cardiac disease by logistic regression analyses.
n = number of successfully genotyped individuals; SNP ID = Identification number of SNP according to the Single Nucleotide Polymorphism database.

Table 3 The effect of common single nucleotide polymorphisms on white matter lesion progression measured as annual white matter lesions load change in the Austrian Stroke Prevention Study cohort and hypertensive individuals

SNP ID	Austrian Stroke Prevention Study cohort			Hypertensives		
	n	β (95% CI)	*P*-value	*n*	β (95% CI)	*P*-value
rs1043994	484	0.087 (0.000 to 0.238)	**0.05**	319	0.136 (0.043 to 0.365)	**0.013**
rs10404382	446	0.082 (−0.018 to 0.235)	0.091	296	0.147 (0.053 to 0.392)	**0.010**
rs10423702	485	0.092 (0.007 to 0.247)	**0.038**	319	0.143 (0.055 to 0.198)	**0.009**
rs1043997	488	0.051 (−0.046 to 0.180)	0.246	320	0.096 (−0.016 to 0.304)	0.078

Significant SNPs are highlighted in bold. β-Coefficients and *P*-values are given for the presence of one minor allele at the corresponding SNPs after adjustment for age, sex, hypertension (only in the entire cohort), diabetes status and cardiac disease by linear regression analyses.
n = number of successfully genotyped individuals; SNP ID = Identification number of SNP according to the Single Nucleotide Polymorphism database.

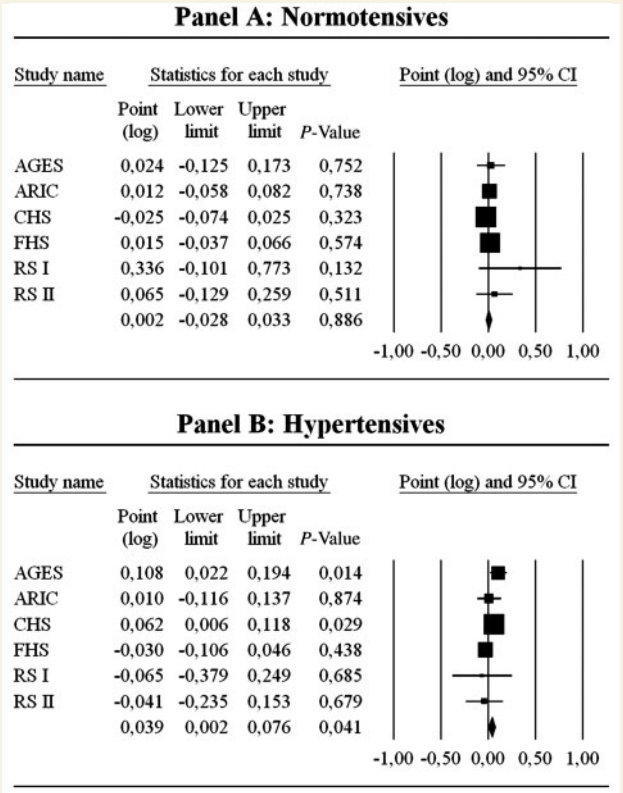

Figure 3 Forest plots for the single nucleotide polymorphism rs10404382 within the CHARGE consortium in normotensives (*Panel A*) and in hypertensives (*Panel B*). Individual studies are plotted against the individual effect sizes (point estimates of the β-coefficients for white matter lesions as continuous trait, log transformed). The size of the box is inversely proportional to the estimated variance of the effect-size estimator. Horizontal lines are the 95% CIs. The diamond represents the meta-analytic effect size. AGES = Aging Gene-Environment Susceptibility-Rejkjavik Study; ARIC = Atherosclerosis Risk in Communities Study; CHS = Cardiovascular Health Study; FHS = Framingham Heart Study; RS = Rotterdam Study.

can be considered specific for CADASIL. This also applies for the lesion at the right temporal lobe because it is located adjacent to the ventricular horn and does not affect the temporopolar white matter, which is strongly associated with CADASIL (Fig. 4A).The subject with the V764A variant showed not only the most pronounced white matter lesion load and progression, but also had slight anterior temporal lobe hyperintensity, considered to be a specific MRI finding in CADASIL cases (Fig. 4A). Another individual with an A1450T exchange did not have anterior temporal lobe hyperintensity, but had three other MRI findings suggestive of CADASIL (Fig. 4B and C). Migraine without aura was reported by one subject carrying the L1518M mutation. The Mini-Mental State Examination score was within normal range in all non-synonymous rare SNP carriers and there was no indication of depression in these individuals. Stroke in first-degree relatives was reported by two subjects with the H170R and S497L mutations.

Functional assessment of rare non-synonymous single nucleotide polymorphisms

We assessed the functionality of the detected non-synonymous rare SNPs by SIFT, PolyPhen2 and by homology modelling and threading (Table 6). Using SIFT, eight amino acid substitutions—H170R, P496L, V764A, H1133Q, V1183M, L1518M, D1823N and V1952M—reached the optimum median sequence conservation values with scores between 0.00 and 0.05 and were predicted to affect the protein function. Also, the A2190V substitution was predicted to be functional but with low confidence. The SIFT scores for the amino acid substitutions S497L, R1262L, A1450T and G1710D, ranged between 0.06 and 0.87 and were predicted as being tolerated. PolyPhen2 predicted H170R, A1450T, L1518M and V1952M as probably damaging, and P496L, V1183M, G1710D and D1823N as possibly damaging to protein function. The method of homology modelling and threading to simulate protein structure alterations upon mutation showed possible functionality for the mutations H170R, P496L, S497L, V1183, R1262L, A1450T, L1518M, D1823N and V1952M (Supplementary Figs 1–9). Due to the different chemical and physical properties of the replaced amino acids, the protein functionality could be altered. Altogether there were six SNPs, H170R, P496L, V1183M, L1518M, D1823N and V1952M, implicated by all three bioinformatical tools as functional. Except for two of these SNPs (V1183M and V1952M), they were exclusively or mainly detected in subjects with severe white matter lesions. Table 6 summarizes the results of the functional SNP assessments.

Discussion

This is the first study investigating the distribution of sequence variations in the *NOTCH3* gene and assessing their role in age-related cerebral small vessel disease in a community-based cohort. We show that *NOTCH3* is highly variable in the elderly with both common and rare SNPs across the gene. Importantly, mutations affecting cysteine residues, a characteristic of CADASIL (Chabriat *et al.*, 2009), were absent in the Austrian Stroke Prevention Study cohort. Four common SNPs in strong linkage disequilibrium significantly enhanced the risk for both the presence and progression of white matter lesions. Importantly, the effect of SNPs on both phenotypes was confined to hypertensives. We found that the SNP, rs10404382, showing the strongest effect on white matter lesions risk in the Austrian Stroke Prevention Study cohort, was also significantly associated with white matter lesions burden in an independent sample of 4773 elderly hypertensive stroke-free individuals of European descent within the CHARGE consortium, including the AGES, ARIC, Cardiovascular Health Study, Framingham Hear Study and Rotterdam Studies I and II. We observed nine rare non-synonymous SNPs, which were only present in subjects with severe white matter lesions. Bioinformatical tools consistently implicated three of these SNPs to be functional. Clinical assessment of the carriers of the nine SNPs did not reveal CADASIL-specific findings. Beside white

Table 4 List and characteristics of rare single nucleotide polymorphisms

SNP ID	cDNA	Allele	Exon	Function	Amino acid change	Domain	Novel	WML$^+$, $n = 195$	WML$^-$, $n = 82$
1	−622	C / T	Prom	5′ near gene	–	–	No	0	1
2	−462	G / C	Prom	5′ near gene	–	–	No	1	0
3	−328	A / C	Prom	5′ near gene	–	–	No	1	3
4	274 + 16	G / A	2	intron	–	–	No	0	1
5[a]	384	G / A	3	NS	R103Q	EGFR 2	Yes	0	1
6	**585**	**A / G**	**4**	**NS**	**H170R**	**EGFR 4**	**Yes**	**1**	**0**
7	847	C / A	5	S	T257T	EGFR 6	Yes	0	1
8	1563	C / T	9	NS	P496L	EGFR 12	No	8	2
9[a]	**1566**	**C / T**	**9**	**NS**	**S497L**	**EGFR 12**	**Yes**	**1**	**0**
10	1581	C / T	10	NS	S502F	EGFR 12	Yes	0	1
11[a]	1717	C / T	11	S	G547G	EGFR 14	Yes	1	0
12[a]	1801	G / A	11	S	T575T	EGFR 14	No	6	1
13	**2367**	**T / C**	**14**	**NS**	**V764A**	**EGFR 19**	**Yes**	**1**	**0**
14	2486 + 452	C / G	15	Intron	–	–	No	2	0
15	3134	G / C	19	NS	A1020P	EGFR 26	No	1	3
16	3475	C / A	21	NS	H1133Q	EGFR 29	No	11	4
17[a]	3623	G / A	22	NS	V1183M	EGFR 30	No	0	2
18	3780	A / T	22	NS	H1235L	EGFR 31	No	3	2
19	**3861**	**G / T**	**23**	**NS**	**R1262L**	**EGFR 32**	**Yes**	**1**	**0**
20	3913 + 11	G / A	23	intron	–	–	Yes	0	1
21	**4424**	**G / A**	**24**	**NS**	**A1450T**	**LNR 2**	**Yes**	**1**	**0**
22	4426	C / T	24	S	A1450A	LNR 2	Yes	1	0
23	**4628**	**C / A**	**25**	**NS**	**L1518M**	**NOD**	**Yes**	**1**	**0**
24	4715	C / G	25	NS	L1547V	NOD	Yes	1	1
25	**5205**	**G / A**	**28**	**NS**	**G1710D**	**TMD**	**Yes**	**1**	**0**
26[a]	**5543**	**G / A**	**30**	**NS**	**D1823N**	**ANK 1**	**Yes**	**1**	**0**
27	5602	T / C	30	S	A1842A	ANK 1	No	0	2
28	5930	G / A	32	NS	V1952M	ANK 5	Yes	2	3
29	6514	A / C	33	S	A2146A	LCR	No	2	3
30[a]	**6645**	**C / T**	**33**	**NS**	**A2190V**	**LCR**	**Yes**	**1**	**0**
31[a]	7045	C / T	33	3′-UTR	–	–	Yes	2	0
32	7138	C / T	33	3′-UTR	–	–	Yes	2	1
33	7245	A / C	33	3′-UTR	–	–	Yes	4	0

Non-synonymous SNPs detected only in individuals with severe white matter lesions are in bold.
a SNPs at CpG sites.
ANK = ankyrin repeat; cDNA = position of the SNP according to the NOTCH3 cDNA sequence, EGFR = epidermal growth factor-like repeat; LCR = low complexity region; LNR = Lin12/Notch repeat; NOD = Notch domain; NS = non-synonymous mutation, prom: promoter; S = synonymous mutation; SNP ID = Identification number of rare SNP according to their position in the NOTCH3 gene; TMD = transmembrane domain; WML$^+$ = individuals with severe white matter lesions, WML$^-$ = individuals with normal MRI.

matter lesions, they showed temporal lobe hyperintensity, hyperintensity in the external capsule, lacunar infarcts and subcortical lacunar lesions in four, the presence of migraine in one and stroke in first-degree relatives of two individuals. Although such findings have been reported in cases of CADASIL, it is important to realize that the lesion at the anterior temporal lobe was not typical for the disease as it did not affect the temporopolar white matter. All other MRI lesion types also occur in elderly subjects without CADASIL. Additionally, subjects with migraine had no aura, which would be more indicative for CADASIL.

Of particular interest is our finding indicating hypertension-dependent effects of *NOTCH3* variants on both prevalence and progression of white matter lesions. It is conceivable that the association is mediated by an impaired vascular response to hypertension due to Notch3 dysfunction. In the adult brain, Notch3 is expressed in the vascular smooth muscle cells of small vessels and it is essential for maintaining normal vascular structure and function (Ayata, 2010; Joutel *et al.*, 2010). Furthermore, it also plays a central role in the autoregulation of cerebral blood flow and cerebral resistance (Joutel *et al.*, 2010). Autoregulation assures that under fluctuating blood pressure, cerebral blood flow remains adequate. The white matter is particularly sensitive to ischaemia (Pantoni *et al.*, 1996) and white matter lesions are thought to be ischaemic manifestations of chronic hypoperfusion with degeneration of myelinated fibres due to repeated selective oligodendrocyte death (Pantoni, 2010). In this context, it is important to note that not only hypertension, but also diurnal blood pressure abnormalities have been shown to be associated with white matter lesions (Gomez-Angelats *et al.*, 2004). NOTCH3 knockout mice showed an enhanced susceptibility to ischaemia

Table 5 Clinical characteristics of non-synonymous rare single nucleotide polymorphism carriers

Amino acid change	ID/sex	Age	Hypertension (SBP/DBP)	Tx	Stroke in first-degree relatives	Anterior temporal lobe hyperintensity	WML type	WML load (cm^3)	WML load progression per year (cm^3)	Hyperintensity of external capsule	Lacunar infarcts	Subcortical lacunar lesions	Migraine	MMSE
H170R	1947/M	66	+(150/100)	–	+	–	Confluent	3	1.43	–	–	–	–	28
S497L	774/M	73	+(138/93)	–	+	–	Early confluent	7	0.25	–	–	–	–	27
V764A	1350/F	71	–(120/85)	–	–	+	Confluent	32	2.96	–	–	–	–	28
R1262L	846/M	76	–(135/75)	–	–	–	Confluent	19	No FU	–	–	–	–	28
A1450T	642/M	70	+(185/90)	–	–	–	Confluent	6	0.50	+	+	+	–	28
L1518M	1271/F	69	+(130/95)	+	–	–	Confluent	12	No FU	–	–	–	+	28
G1710D	1538/F	78	+(140/80)	–	–	–	Confluent	8	1.46	–	–	+	–	24
D1823N	846/M	76	+(135/75)	–	–	–	Confluent	19	No FU	–	–	–	–	28
A2190V	144/M	66	+(185/98)	–	–	–	Early confluent	19	No FU	–	+	–	–	30

DBP = mean diastolic blood pressure at examination; FU = follow up; HT = hypertension; ID = identification number of study participants, MMSE = Mini-Mental State Examination, SBP = mean systolic blood pressure in mmHg, Tx = on antihypertensive therapy, WML = white matter lesions.

(Arboleda-Velasquez *et al.*, 2008). Notch3 is also involved in the local vascular reaction to mechanical stress and to hypertension by modulating vascular smooth muscle cell phenotype and by affecting the balance between their proliferation and apoptosis (Morrow *et al.*, 2005, 2008). The observed hypertension dependent effect of *NOTCH3* variants is opposite to our earlier observations on the angiotensinogen promoter B haplotype, which affects the risk for white matter lesions independently of the presence of hypertension (Schmidt *et al.*, 2001), probably by leading to an over-expression of angiotensinogen in astrocytes (Schmidt *et al.*, 2004). Astrocytes and vascular smooth muscle cells are major constituents of the neurovascular unit, and thus these findings support a possible role of this structure in the development of age-related white matter lesions.

Although the role of Notch3 in the development of age-related cerebral small vessel disease can easily be envisioned, it remains speculative how the described mutations alter its function. The common SNPs associated with white matter lesions are synonymous or intronic mutations and are in strong linkage disequilibrium with each other. They are most probably not causal themselves but rather mark the presence of yet undetected functional mutation(s) in linkage disequilibrium. On the other hand, recent evidence also suggests that synonymous mutations are able to alter translational efficiency of a gene and, therefore, might be pathogenic (Berleant *et al.*, 2009).

Typically, CADASIL mutations are located within epidermal growth factor-like repeats, they cluster in exons 3 and 4 and are characterized by an uneven number of cysteine residues. It has been argued that CADASIL mutations are most probably gain of function mutations because they occur in regions with high sequence diversity and low evolutionary conservation and because of their stereotyped nature (Donahue and Kosik, 2004) although a recent report rather supports a hypomorphic nature at least for two CADASIL mutations (Arboleda-Velasquez *et al.*, 2011). Possible new functions acquired by the mutations are misfolding of the protein leading to aggregation or impaired protein–protein interactions. Contrary to the stereotypic nature of CADASIL mutations and their clustering in epidermal growth factor-like repeats, the 42 different point mutations we detected in the normal elderly are dispersed over the whole gene and the non-synonymous changes lead to diverse amino acid substitutions. This finding is more supportive for loss of function or hypomorphic mutations. This is also in line with the observed gene dose effect for the risk alleles. Non-cysteine (R75P) mutation in Korean and Japanese CADASIL patients with granular osmiophilic material depositions have been previously reported (Kim *et al.*, 2006; Muzino *et al.*, 2008). These patients manifested multiple cerebral infarctions and white matter lesions, which importantly did not extend to the temporal lobe. The authors hypothesized that the R75P mutation lead to a destabilization of the adjacent disulphide bridges between the cysteine residues. Recently, another non-cysteine amino acid substitution, L1515P, has been described in a French patient with cerebral small vessel disease lacking granular osmiophilic material deposits, and Notch3 extracellular domain accumulation. This mutation led to a destabilization of the Notch3 heterodimer and to a constitutive activation of the Notch3 receptor (Fouillade *et al.*, 2008). This mutation is located within a

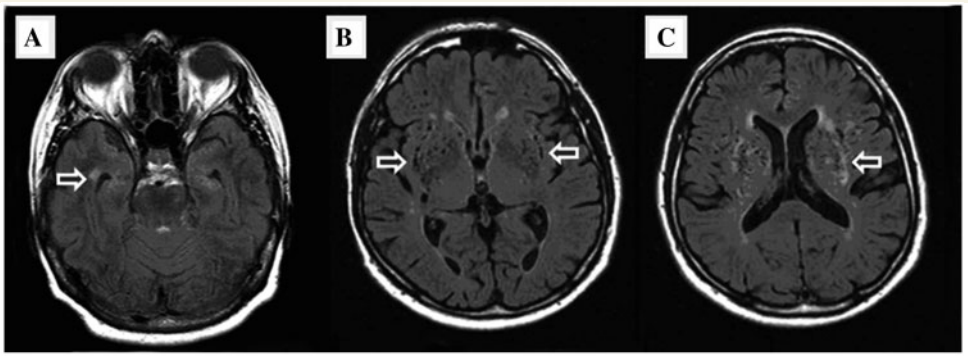

Figure 4 MRI findings suggestive of CADASIL in carriers of rare non-synonymous single nucleotide polymorphisms. Axial fluid-attenuated inversion recovery MRI images. (**A**) Subject 1350 with a V764A amino acid exchange showing a hyperintense rounded area of signal hyperintensity in the temporal lobe directly adjacent to the temporal horn of the lateral ventricle (*arrow*). Although signal changes at this location are not typically seen with age-related white matter changes, the abnormality can also not be considered specific for CADASIL, because CADASIL lesions usually extend to the most anterior parts of the temporopolar white matter. (**B**) Subcortical lacunar lesions which have been reported with CADASIL (van den Boom *et al.*, 2002) are shown in the subinsular region and operculum of the frontal lobe bilaterally. (**C**) demonstrates confluent hyperintensity of the external capsule (*arrow*), a finding that has high sensitivity, but low specificity in CADASIL (Markus *et al.*, 2002).

Table 6 Predicted consequences of amino acid substitutions on protein function of the non-synonymous rare single nucleotide polymorphisms observed only in individuals with severe white matter lesion by SIFT and Polyphen2 analysis and with homology modelling and threading

Exon	Amino acid change	Domain	Prediction SIFT	Prediction polyphen2	Molecular modelling
3	R103Q	EGFR 2	Tolerated	Benign	No significant structural change
4	**H170R**	**EGFR 4**	**Affected**	**Probably damaging**	**Additional H-bond**
9	**P496L**	**EGFR 12**	**Affected**	**Possibly damaging**	**Possible stronger van der Waals interactions**
9	S497L	EGFR 12	Tolerated	Benign	Possible change in EGFR folding
10	S502F	EGFR 12	Tolerated	Benign	No significant structural change
14	V764A	EGFR 19	Affected	Benign	No significant structural change
19	A1020P	EGFR 26	Tolerated	Benign	Predicted structure is not reliable
21	H1133Q	EGFR 29	Affected	Benign	No significant structural change
22[a]	V1183M	EGFR 30	Affected	Possibly damaging	Possible EGF-EGF interaction
22	H1235L	EGFR 31	Tolerated	Benign	Predicted structure is not reliable
23	R1262L	EGFR 32	Tolerated	Benign	may influence EGFR-EGFR folding
24	A1450T	LNR 2	Tolerated	Probably damaging	Additional H-bond
25	**L1518M**	**NOD**	**Affected**	**Probably damaging**	**Disruption of the leucine zipper pattern**
25	L1547V	NOD	Tolerated	Benign	No significant structural change
28	G1710D	TMD	Tolerated	Possibly damaging	No results
30	**D1823N**	**ANK 1**	**Affected**	**Possibly damaging**	**Possible ANK-ANK interaction**
32[a]	V1952M	ANK 5	Affected	Probably damaging	Additional H-bond
33	A2190V	LCR	Tolerated	Benign	No results

a SNPs not supported clinically but by all three bioinformatic tools; SNPs supported both clinically as well as by all three bioinformatic tools are given in bold.
ANK = ankyrin repeat; EGFR = epidermal growth factor-like repeat; prom: promoter; LCR = low complexity region; LNR = Lin12/Notch repeat; NOD = Notch domain; TMD = transmembrane domain.

leucine zipper motif of the Notch domain close to the L1518M mutation detected in our cohort. Seven of the nine rare SNP carriers showed some 'CADASIL-like' clinical signs or MRI features, which further supports functionality for the SNPs H170R, P496L, L1518M and D1823N. Importantly, none of the rare SNP carriers showed all characteristic symptoms of CADASIL, probably indicating the central role of cysteine mutations in this disease. Although the individual with the D1823N did not present any CADASIL-like signs, she had one of the most severe white matter lesions and this mutation was predicted by all three *in silico* methods as functional. The clinical characteristics of the R1262L carrier were similar, but for this mutation *in silico* analyses were ambiguous. In addition to the deepening of our understanding about the pathogenesis of age-related cerebral small vessel disease, knowledge of

the mutation spectrum at *NOTCH3* in the normal elderly should also facilitate the interpretation of atypical non-cysteine mutations in CADASIL diagnostics.

The strengths of our study are that it investigates for the first time a community based, normal elderly population for the presence of *NOTCH3* gene variations and provides a comprehensive list of SNPs in Europeans over the promoter, all exons and 3′-untranslated regions. Extensive phenotyping and the availability of follow-up data in the Austrian Stroke Prevention Study cohort allowed the investigation of the effect of mutations both cross-sectionally and in longitudinal fashion. Further on the association of the common SNP, rs10404382, with the strongest effect on white matter lesions in the original cohort was replicated in a large sample of elderly individuals of European descent. Further investigation of this SNP (or rs1043994 and rs10423702 that are in strong linkage disequilibrium with it) in different cohorts and ethnic groups are warranted to solidify their relevance in white matter lesions risk. The functional relevance of the reported rare mutations is still speculative and further studies on relatives of the carriers as well as experimental studies are needed to establish causality.

A potential problem of our study is that we have performed multiple statistical tests in order to explore whether genetic variations at the *NOTCH3* gene are relevant in age-related cerebral small vessel disease. We investigated two primary phenotypes, white matter lesions and lacunes and the secondary phenotype white matter lesions progression. We investigated two statistical models in the regression analyses, once we adjusted for age and then for additional possible confounders including sex, hypertension, cardiac disease and diabetes to see if the effect size was changed. We also tested nine common SNPs for associations with white matter lesions and then followed up on the most significant four, which were highly correlated ($R^2 > 0.9$). Finally, we explored the association in two subgroups by stratifying on hypertension. In the results section, we did not adjust for multiple testing, as both the outcome measures and the predictive SNPs are correlated, and an adequate adjustment is therefore difficult. Although due to multiple testing, the possibility of false positive findings in the discovery sample is enhanced, it should be noted that the proportion of statistically significant findings was much $>5\%$, which one would expect by chance. Nevertheless, the most important support for the assertion that findings in the Austrian Stroke Prevention Study cohort are not due to chance comes from the replication of our findings both in the individual cohorts AGES and Cardiovascular Health Study as well as in meta-analyses of large independent samples of stroke-free individuals within the CHARGE consortium.

In summary, our study shows that the *NOTCH3* gene is highly variable in the elderly and that common variants at the *NOTCH3* gene increase the risk of age-related white matter lesions in the presence of hypertension. Additional investigations are required to identify the mode of action of described *NOTCH3* variants. Although conceivable, it needs to be explored as to whether Notch3 signalling *per se* might be a relevant pathway in the development of cerebral small vessel disease with advancing age.

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Supplementary material

Supplementary material is available at *Brain* online.

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