HHS Public Access

Author manuscript

Stroke. Author manuscript; available in PMC 2019 June 01.

Published in final edited form as:

Stroke. 2018 June ; 49(6): 1557–1562. doi:10.1161/STROKEAHA.117.017073.

Imaging Endophenotypes of Stroke as a Target for Genetic Studies

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Keywords

stroke; genetics; brain MRI; white matter hyperintensities; imaging

Subject terms

Genetic; Association Studies; Cerebrovascular Disease/Stroke

Introduction

Stroke is a heterogeneous disease leading to death of neural tissue and often resulting in the loss of motor and cognitive function. It is the fifth leading cause of death and a leading cause of severe long-term disability in the United States.[1] Most strokes (~80-90%) are caused by an acute interruption of the brain arterial blood supply due to vascular occlusion, leading to brain tissue ischemia. Approximately 10-20% of strokes are caused by blood vessel rupture, leading to hemorrhage. While a growing number of genetic loci have been identified for the major stroke risk factors, the genetic architecture of stroke and its subtypes remains largely uncharacterized. The use of imaging measures as endophenotypes in genetic studies of stroke is leading to new discoveries and may provide a better understanding of the biological mechanisms underlying stroke etiology.

The concept of endophenotype was first developed in the early 1970's by Gottesman and Shields for schizophrenia research.[2] An endophenotype was originally defined as a quantitative characteristic of the disease, which cannot be observed by the naked eye ("Endo-" means internal or inside; "Pheno-" means showing or appearing).[3] It is not a risk factor but rather an expression of the underlying disease liability. Gottesman and Gould identified six criteria that define an endophenotype (Table 1).[3] Because endophenotypes are typically quantitative and lie in the causal pathway to the disease but are closer to the gene action than the clinical phenotype,[3] they provide greater power than their corresponding clinical phenotypes in gene discovery, as has been shown in the genetic study of other

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Disclosures

None

complex diseases.[4, 5] This review will discuss selected imaging endophenotypes of stroke. Genetic studies referenced in this article mostly focus on genome-wide association studies (GWASs) in large population-based samples.

Brain MRI Endophenotypes of Cerebral Small Vessel Disease

Cerebral small vessel arteriopathy manifests itself as heterogeneous lesions in the brain parenchyma detectable by MRI, including white matter hyperintensities, infarcts or lacunes, dilated perivascular spaces, and cerebral microbleeds.[6]

White matter hyperintensities (WMH) are common neuroradiological abnormalities in the elderly and are detectable by T2-weighted (T2W) and fluid attenuation inversion recovery (FLAIR) structural MRI. WMH have been widely recognized as significant predictors of stroke, dementia, and mortality.[7] Genetic factors play a significant role in WMH susceptibility, with heritability estimates ranging from 55% to 80%.[8–10]. A GWAS of WMH burden conducted in middle-aged to elderly individuals who were free of dementia and stroke and were from community-based cohorts identified a locus on chromosome 17q25 near *TRIM47*.[11] This finding has been confirmed in several independent studies[12–14] and in a subsequent, expanded GWAS including participants of European, African, Hispanic, and Asian descent.[15] In the latter GWAS, four novel loci were also identified on chr10q24 (*PDCD11/NEURL/SH3PXD2A*), chr2p21 (*HAAO*), chr1q22 (*PMF1*) and chr2p16 (*EFEMP1*).[15] Remarkably, 4 of the 5 loci encompass genes that have been implicated in tumors of the glial cells, including gliomas, astrocytomas, and glioblastomas, suggesting that inflammatory and glial proliferative pathways may be involved in the development of WMH in addition to previously-proposed ischemic mechanisms. Interestingly, chr1q22 (*PMF1*) has also been identified as a risk locus for non-lobar intracerebral hemorrhage[16], as well as all stroke and ischemic stroke[17]; and chr10q24 (*SH3PXD2A*) has been identified in a recent GWAS of all stroke[17], further demonstrating the utility of neuroimaging endophenotypes in the search for stroke genes. A genetic risk score constructed from 18 SNPs most significantly associated with WMH in the latest multi-ethnic GWAS[15] was recently evaluated for association with lacunar stroke, cardioembolic stroke, and large vessel stroke in cases obtained from hospital admissions in Europe and Australia and ancestry-matched controls.[18] There was strong evidence that genetic variants associated with WMH also influence risk of lacunar stroke but not that of other stroke subtypes, supporting the notion that the two disorders share common pathological processes possibly affecting the small vessels of the brain.

A recent GWAS of WMH in 3,670 stroke patients from the United Kingdom, United States, Australia, Belgium, and Italy did not identify genome-wide significant loci.[18] However, meta-analyses of results with those derived from the large GWAS from the CHARGE consortium[15] identified 4 novel loci reaching genome-wide significance: rs72934505 (*NBEAL1*); rs941898 (*EVL*); rs962888 (*EFTUD2/C1QL1*); and rs9515201 (*COL4A2*). Interestingly, *COL4A2* and the adjacent *COL4A1*, encode alpha subunits of type IV collagen the major structural component of basement membranes and have been implicated in hereditary cerebral small vessel disease and intracerebral hemorrhage.[19, 20]

Because common variants detectable by GWAS have been estimated to account for at most a quarter of the WMH phenotypic variance[21], genetic studies of WMH are now focusing on rare variants and other "omics".[22] An analysis of 250,000 mostly rare to low frequency variants, mapping to coding regions of the genome (exome) and genotyped in 20,719 participants of European and African ancestry, showed that rare non-synonymous variants in *MRPL38*, located in the previously identified chr17q25 locus, are associated with WMH independently of the known GWAS signal (manuscript submitted). *MRPL38* encodes a mitochondrial ribosomal protein. Gene mutations resulting in impaired mitochondrial translation have been implicated in severe, early onset neurological disease.[23] Future whole genome sequence analysis will provide a more complete picture of the role of rare variants in WMH susceptibility.[24]

MRI-defined brain Infarcts (BI) are common in the elderly and typically occur in the absence of clinically recognized stroke symptoms. Like WMH, they are associated with future incident cognitive decline and stroke. The majority (>90%) of BI are small subcortical brain infarcts (SSBI) 3 to 15 mm in size, which are also referred to as *lacunes*. The remaining 10% are larger subcortical infarcts or cortical infarcts.[25] A GWAS of covert MRI infarcts in 9,401 participants from 6 community-based cohorts (mean age: 69 years; 19.4% had at least one MRI infarct) identified novel associations in the *MACROD2/FLRT3* region of chromosome 20p12.[26] A more recent trans-ethnic meta-analysis of GWAS in 20,949 participants from 5 ethnicities has been completed. Both MRI-defined BI (N=3,726) and SSBI (N=2,021) were analyzed. Two loci reached genome wide significance for association with BI: *FBN2* and *LINC00539/ZDHHC20*. However, associations were not replicated in a smaller independent sample of 3,143 participants, including 1,134 with BI and 543 with SBBI. The inconsistent findings among studies and the failure of replication efforts may not only be attributable to insufficient power, genetic heterogeneity across ethnic groups and the use of different definition of BI (e.g., diameter threshold may vary across studies) may also in part explain these observations. These loci did not appear to associate with ischemic stroke and pathologically defined BI, warranting additional studies to validate these findings and further examine the shared etiology between covert and overt brain vascular disease.[22]

Recent application of high resolution structural MRI and ongoing development of semi-automated detection techniques have allowed assessment of cortical cerebral *microinfarcts* (diameter <1 mm).[27] The cause of these brain abnormalities is likely heterogeneous. Cerebral microinfarcts have been associated with dementia, cognitive decline, and motor function impairment.[28] The genetic basis of cerebral microinfarcts is unknown.

Perivascular spaces (also known as Virchow–Robin spaces) are fluid-filled spaces that follow the typical course of penetrating vessels through the brain parenchyma. They appear either linear if imaged parallel to the course of the vessel or round or ovoid (diameter < 3 mm in general) if imaged perpendicular to the course of the vessel, with hyperintense signal on T2 images.[29] Enlarged perivascular spaces (EPVS) have recently emerged as markers of cerebral small vessel disease.[29, 30] The causes of EPVS remain unclear but impaired blood–brain barrier might play a role.[31] A genetic basis for EPVS was suggested by recognition of a familial occurrence perivascular space dilations in children with neurodevelopmental abnormalities.[32] However, no large-scale GWAS of EPVS has been reported to date. In a

two-stage imaging genetic study of Attention-Deficit Hyperactivity Disorder in 2,875 children from the general population aged 7 to 10 years, rs273342, a SNP mapping to *MAPRE2* at 18q12.1 locus, was significantly associated with EPVS in the basal ganglia.[33] Large-scale studies such as GWASs are warranted to increase the power to uncover the genetic architecture of EPVS and understand its relationship to small vessel stroke.

Cerebral microbleeds (CMBs) are small, round, or ovoid perivascular hemosiderin deposits detectable by T2* Gradient-Recall Echo (GRE) or Susceptibility-Weighted (SWI) MRI. They occur in 5% to 21% of apparently healthy people, but are more frequent in patients with ischemic stroke (30-40%) or with intracerebral hemorrhage (ICH) (>60%).[34] CMBs located in deep subcortical or infratentorial regions are typically associated with hypertensive vascular disease, while those located in lobar regions are usually associated with cerebral amyloid angiopathy.[35] A systemic review and meta-analysis of candidate gene associations showed that the *APOE* ε4 allele is associated with a higher risk of microbleeds at any location but the association was stronger with strictly lobar CMBs.[36] In a GWAS of ICH, *APOE* ε4 was associated with both lobar and deep ICH.[37] To date, no GWAS of microbleeds has been reported.

White matter microstructure and Cerebral Small Vessel Disease

White matter microstructure can be assessed by diffusion tensor imaging (DTI). Fractional anisotropy (FA) and mean diffusivity (MD) are commonly used DTI metrics to quantify changes in white matter integrity that are not typically detected on conventional MRI.[38] These changes have been shown to represent early predictors of the course of age-related white matter degeneration with time and to precede irreversible white matter lesions (WMH).[39, 40] In the Rotterdam study, a large population-based cohort of middle-aged and older adults, measures of white matter microstructural integrity were associated with an increased risk of stroke, independent of cardiovascular risk factors and MRI-defined white matter lesions and lacunar infarcts.[41] Heritability of whole brain FA and regional FA was recently estimated to range between 0.66 and 0.90.[42] A GWAS of global FA in a sample of 776 Mexican-American members of extended pedigrees identified 5 mostly intergenic loci but no replication was attempted as part of this study.[43] Additional genetic studies in larger samples are needed to uncover the genetic basis of white matter integrity and its role in stroke susceptibility.

Carotid Ultrasound Imaging Endophenotypes of Large Artery Stroke

Carotid atherosclerosis, as a source of microemboli or a cause of ischemia due to flow limiting stenosis, is responsible for 15-20% of strokes. *Carotid intima media thickness* (cIMT) and *carotid plaque burden* can be assessed using non-invasive high resolution ultrasound techniques.[44] cIMT reflects the thickening of the inner two layers of the carotid artery wall – the intima and media, and plaque represents established atherosclerotic disease in the lumen. Asymptomatic carotid stenosis (ACS) is common in the general population, with prevalence estimated at up to 7.5% for moderate ACS and up to 3.5% for severe ACS.[45] Presence of carotid artery plaque may be even higher, ranging between 15-35% of adults.[46, 47] Both measures have been associated with an increased risk of future stroke[48, 49] and

are influenced by genetic factors.[50] A GWAS of subclinical atherosclerosis in over 40,000 individuals of European ancestry identified three genome-wide significant loci associated with cIMT (8q24: *ZHX2*; 19q13: *APOC1*; 8q23.1: *PINX1*) and two with carotid plaque (7q22: *PIK3CG*; 4q31: *EDNRA*).[50] In the largest GWAS of stroke to date, the *EDNRA* locus was significantly associated with large artery stroke, exclusive of other stroke subtypes.[17] These findings underscore the utility of endophenotypes for uncovering possible biological mechanisms of stroke pathophysiology.

Endophenotypes of Cardioembolic and Cryptogenic Stroke

Cardioembolic stroke (CES) accounts for 15-30% of all ischemic strokes, while undetermined (cryptogenic) stroke accounts for 30-40%.[51, 52] Atrial Fibrillation (AF), the most common cardiac arrhythmia, is a major cause of CES.[53] To date, at least 30 genetic loci have been associated with AF.[54] Two of these are among the first and most consistently identified ischemic stroke loci (4q25: *PITX2* and 16q22: *ZFHX3*), with *ZFHX3* exclusively associated with CES.[55] Thus, information about the genetic architecture of AF may be relevant to stroke. This is also supported by the reported association of a comprehensive AF polygenic risk score with stroke, more specifically CES.[56]

Thrombus formation in the left atrial appendage (LAA) in patients with AF is the most common cause of cardioembolic events.[57] *Measures of LAA structure and function* can be assessed by echocardiography (transesophageal echocardiography (TEE) or transthoracic echocardiographic (TTE)) and may represent valuable endophenotypes of cardioembolic or cryptogenic stroke.[58] Three anatomical features of the LAA have been associated with increased risk of ischemic stroke in patient with AF: shape, orifice size, and fibrosis.[59] In addition to LAA structure, LAA function, commonly assessed by echocardiographic measurement of LAA blood flow velocities, has also been correlated with stroke risk, with lower velocities associated with greater ischemic stroke risk and thrombus formation.[59] Studies are needed to investigate the genetic basis of LAA structure and function and the possible role of the underlying genes in determining ischemic stroke risk, particularly cardioembolic and cryptogenic stroke.

Recent data indicate that left atrial thromboembolism can occur even in the absence of AF. Hence, a broader concept considering atrial dysfunction, or atrial cardiopathy, as a stroke risk factor has been recently proposed.[60] Biomarkers of atrial cardiopathy may, thus, represent valuable endophenotypes of stroke, especially embolic stroke.

Left atrial size measured by echocardiography has been associated with age-adjusted ischemic stroke risk in the Framingham Heart Study[61] and in the ethnically diverse Northern Manhattan Stroke Study (NOMASS).[62] Linkage analysis in 100 Caribbean Hispanic families followed by association analysis in 825 participants from NOMASS identified a region on chr.17p10 harboring multiple susceptibility genes implicated in heart structure (*NTN1, MYH10, COX10, and MYOCD*).[63] In a recent meta-analysis of GWAS of left atrial size from 21 studies with up to 30,201 individuals, no association reached genome-wide significance for left atrial size.[64] The strongest association was with rs2292864 ($P=5.15\times10^{-7}$), located in an intron of *ITGB3*, encoding the beta chain beta 3 of integrin, a

cell-surface receptor involved in cell adhesion and cell-surface mediated signaling. Whether genetic variants in genes implicated in left atrial size to date are associated with stroke has not been examined.

Increased P-wave terminal force in lead V1 (PTFV1) is the most commonly used electrocardiographic marker of left atrial abnormality, including left atrial fibrosis, elevated filling pressures, and dilatation.[65] In a recent meta-analysis, increased PTFV1, defined either as a continuous or categorical variable, was associated with an 18-22% increased risk of incident ischemic stroke.[66] In the NOMASS, there was no association of PTFV1 with noncardioembolic stroke subtypes.[67] Similarly, in the Atherosclerosis Risk in Communities studies, this association was limited to non-lacunar stroke.[68] To our knowledge, no genetic studies of PTFV1 have been carried out to date. Of note, a newly-identified genetic locus for CES, *NKX2-5*, has also been previously reported associated with ECG-defined PR interval, a marker of atrial and atrioventricular nodal conduction,[69] but not consistently with AF.[17] This result further highlights the relevance of identifying genes underlying atrial cardiopathy to understand stroke pathogenesis.

Integrating multiple endophenotypes to study the genetics of stroke

Investigating stroke endophenotypes individually may not provide the most efficient or powerful approach to dissecting the genetic architecture of stroke. Because many endophenotypes have a vascular origin, they tend to be correlated with each other, some are even highly correlated. For example, microbleeds are associated with SSBI independently of WMH;[70] most incident lacunes are located at the edge of WMH.[71] Thus, a Bonferroni correction applied to multiple GWASs of correlated traits may be too conservative and may reduce statistical power. In addition, while endophenotypes of SVD are generally correlated, only one (or few) endophenotype may reflect a specific etiological component of the disease rather than capturing the global effect of disease on the brain. The recent discovery of the *FOXF2* locus via GWAS of incident all stroke, regardless of subtype, suggest that there may be an advantage in applying strategies of gene discovery that target global mechanisms of brain dysfunction.[72] To increase the power to identify genetic variants associated with stroke, efforts should be made in (1) developing statistical methods accounting for correlation among traits, such as multivariate methods or the combination of univariate analyses results correcting for phenotypic correlations;[73] or (2) integrating multiple specific endophenotypes into a common one, either binary or ordinal. For example, Klarenbeek et al. developed a simple ordinal total SVD score ranging from 0-4 by counting the presence of four SVD endophenotypes in an individual: lacunes, WMH, perivascular spaces, and microbleeds.[74] Various studies showed that a higher score predict stroke severity[75, 76] and cognitive decline[77–79] and is associated with SVD risk factors such as hypertension, age, and smoking.[74, 80, 81] These data indicate that total SVD score may be a better measure of the total SVD burden than individual measures and, thus, could be used in large-scale genetic studies not only to increase statistical power but also to ease the burden of multiple testing. However, the ordinal score only considers four of the SVD endophenotypes and assigns equal weight to each one, thus leaving sufficient room for improvement, e.g., by including additional components such as SSBI and brain atrophy, or weighting each trait differentially

based on its prevalence in the population, the location in the brain, or the number and size of the damage.

Conclusion and Perspectives

Genome-wide genetic studies of stroke conducted to date have required large sample sizes and have yielded a limited (albeit growing) number of loci in comparison to other complex diseases. Genetic and phenotypic heterogeneity pose a major challenge to the elucidation of the genetic basis of stroke. Endophenotypes such as those described above represent a prospect for understanding stroke pathophysiology, improving stroke diagnosis, and developing therapeutic targets for stroke treatment. The ability to measure them quantitatively, reliably, and non-invasively in large population samples provides an opportunity to improve statistical power to detect small genetic effects over that of the binary disease trait. So far, use of stroke endophenotypes has been successful in the discovery of genes associated with WMH, whereas more work remains to be done for others. Different yet related measures can be used to study the mechanisms of different stroke subtypes or uncover global mechanisms common to all stroke subtypes. Stroke gene discovery may also be benefit from integrating multiple endophenotypes into a composite measure or employing longitudinal design to assess genetic underpinnings of disease progression.

Acknowledgments

Sources of Funding

The authors are supported in part by grant R01-NS087541 from the National Institutes of Health.

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Table 1

Criteria defining an endophenotype[3]

1. The endophenotype is associated with the disease in the population
2. The endophenotype is heritable
3. The endophenotype is primarily state-independent (can be measured in both affected and unaffected)
4. Within families, endophenotype and disease co-segregate.
5. The endophenotype measured in affected family members is present in non-affected family members at a higher rate than in the general population
6. The endophenotype is a trait that can be measured reliably, and ideally is more strongly associated with the disease of interest than with other related conditions