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Background and Purpose—A large number of candidate gene association studies have attempted to identify genes implicated in stroke, but there have been few replicable and robust associations reported.

Summary of Review—The skantness of replicable associations partly relates to poor study design. Important methodological considerations include adequate sample size, the selection of appropriate controls, careful clinical phenotyping using standardized classification systems, and determining associations with stroke subtypes as well as stroke as a whole. The use of intermediate phenotypes, particularly carotid intima-media thickness and MRI white matter hyperintensities, appears promising. It is essential that positive associations are replicated in independent populations, and appropriate methodology is used in such studies. To be of use for others, association studies should meet certain standards. In particular, genetic association studies should enable replication studies and meta-analyses.

Conclusions—This article discusses key methodological aspects and suggests standard criteria for candidate gene studies in stroke. *(Stroke. 2005;36:2027-2031.)*

Key Words: genetics □ stroke

Genetic factors appear to be important in multifactorial stroke pathogenesis, but the underlying molecular basis remains uncertain. Over the last decade, increasing numbers of studies investigating associations between DNA sequence variants and stroke have been published, and this trend is likely to accelerate. Increasingly rapid and cheaper genotyping technologies are becoming available, allowing large numbers of polymorphisms to be determined. As a consequence, the dimensions of studies and complexity of the data will increase.

There have been many reports of “significant” associations, but the majority have not been replicated. This has raised concerns about association studies and complex genetics in general. Because of such concerns, a number of journals have proposed guidelines for reporting genetic association studies. *(Stroke)* does not have such formal guidelines. Nevertheless, there are a number of methodological aspects that are important in planning and interpreting such studies in stroke. This article addresses these issues, provides recommendations on how to optimize study design, and suggests a set of standard criteria for genetic association studies in stroke.

Defining the Target Phenotype

The biological mechanisms underlying specific stroke subtypes differ substantially, and specific mutations or polymorphisms may contribute selectively to the risk of a particular stroke subtype. The classification systems for categorizing stroke subtypes vary between studies. The most widely used systems are the Trial of Org 10172 in Acute Stroke Treatment (TOAST) and Oxfordshire Community Stroke Project (OSCP) system. The OSCP system uses clinical examination rather than investigations to classify into clinical syndromes; it is not a pathophysiological classification and is therefore less suited to investigating risk factors for specific subtypes. Pathophysiological classification systems such as TOAST are more appropriate, although their accuracy is dependent on the quality of the clinical information and the experience of the raters. Therefore, the proportion of patients receiving a computed tomography (CT) scan, MRI, imaging of the cerebral vasculature, echocardiography, and other investigations is important. Using predefined phenotyping protocols and central rating by specially trained raters improves the quality of stroke categorization and helps to identify biologically meaningful and reproducible stroke subtypes.

Certain genes, such as those influencing neuronal responses to injury, may predispose to all forms of stroke. Therefore, looking for genetic associations with stroke in general remains a justifiable approach and has successfully been used in the past to identify gene loci and genes implicated in stroke risk. Nevertheless, accurate phenotyping and performing separate analyses according to stroke subtypes are essential. Focusing on particular stroke subtypes...
may make a study more efficient and may markedly reduce necessary sample sizes.\textsuperscript{2} Another way of increasing power may be to focus on early-onset cases, as would be expected for any genetic predisposition. Family history studies suggest the genetic component is stronger in this group. That this is not attributable to recall bias is supported by a prospective twin study.\textsuperscript{12}

Instead of looking at the end point of stroke itself, several groups have focused on intermediate phenotypes. The 2 most widely used are carotid intima-media thickness (IMT) and MRI white matter hyperintensities (WMHs) as intermediate phenotypes for large artery and small-vessel stroke, respectively. Focusing on intermediate phenotypes has a number of advantages: (1) they represent an intermediate stage in disease pathogenesis, and therefore, fewer genes may contribute making the system less complex; (2) they are relatively easy to obtain and can be measured with high accuracy; (3) they are quantitative traits that allow more powerful statistics to be applied; (4) they can be obtained in population-based samples, enabling large sample sizes to be more easily obtained; and (5) they overcome the problem of subclinical disease in a case control study in which the control may be attributable to develop stroke shortly after recruitment.

Carotid IMT and WMH volume has a strong genetic component.\textsuperscript{13–15} Carotid IMT is an independent predictor of stroke, and studies have found associations between a variety of candidate genes and increased IMT. Some of them have been replicable and have emphasized the importance of gene–environment interactions.\textsuperscript{16} Whether increased IMT represents atherosclerosis, remodeling, or a combination remains controversial. Therefore, positive associations should be replicated in populations with the end point of large vessel stroke. Neuropathological–radiological correlations have shown that confluent WMHs on MRI represent small vessel disease,\textsuperscript{17} and this phenotype has been used successfully in association studies investigating the renin-angiotensin system.\textsuperscript{18} However, WMHs are much more common than small vessel stroke, and therefore, positive associations need to be replicated in this stroke group.

Recent studies have suggested the importance of gene–environment interactions in stroke and other cardiovascular disease, for example, between smoking and proinflammatory cytokine genotypes.\textsuperscript{19} Identifying such interactions requires careful recording of conventional risk factors and large sample sizes to identify associations within subgroups.

**Genetic Approach**

There are 2 principle approaches to the identification of disease genes: linkage analysis and association studies.

Linkage analysis rests on the coinheritance of loci that lie near each other on the same chromosome. As a consequence, linkage studies require DNA and clinical information from >1 family member. Linkage analysis has been used successfully in monogenic disorders such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)\textsuperscript{20} and recently in stroke as a complex trait.\textsuperscript{10} Yet, there are a number of challenges to its application in complex diseases.\textsuperscript{21–23}

Association studies compare the frequency of specific DNA sequence variants (alleles) in groups of individuals in a case-control design. An allele is said to be associated with the disease if its frequency differs between cases and controls more than would be predicted by chance. For complex traits, association studies are more efficient than linkage studies because they have greater statistical power to detect several genes of small effect.\textsuperscript{21} However, they also pose important methodological challenges.

An important consideration is the selection of appropriate controls. This must be from a genetically similar population to avoid the introduction of bias attributable to population stratification. The prevalence of some potential stroke risk polymorphism varies markedly between ethnic groups,\textsuperscript{24} making matching cases and controls for ethnicity important. Defining ethnicity may be difficult in populations with marked admixture, and some groups recommend recording ethnicity and geographic origin of parents as well as cases. However, even when efforts are made to match for ethnicity, unrecognized population structure can cause false associations and failure to detect genuine associations. Statistical methods have been proposed to account for population structure so that association studies can proceed even when population stratification is present.\textsuperscript{25–27} One way to avoid population stratification is to use family-based controls. The transmission disequilibrium test (TDT) was developed to match cases and controls on their immediate genetic background: the parents. In late-onset conditions such as stroke, parents are often not alive, making TDT unrealistic.\textsuperscript{28} The sibling TDT, which requires sibs to be recruited, is potentially feasible in stroke but is still a major undertaking.\textsuperscript{28}

Another challenge lies in the selection of target genes and sequence variants. Up to now, association studies have been limited to the investigation of candidate genes selected because of a priori hypotheses about their etiological role in a disease. Thus, these studies depend on the ability to predict functional candidate genes and polymorphisms. There are strategies to optimize and prioritize their selection.\textsuperscript{29} Candidate genes may be selected from regions that have been identified through genomewide scans.\textsuperscript{10,11,30} Alternatively, genes may be prioritized because they are known to encode for specific proteins related to the disease process. Criteria for the selection of specific polymorphisms include the type of polymorphism and its frequency in the population.\textsuperscript{29} As a rule, alleles with frequencies of \( \geq 5\% \) are necessary. Lower allele frequencies pose a challenge to sample size (Table 1). Recently, investigators have begun performing large-scale association studies involving multiple candidate genes and polymorphisms at a time. This can introduce the problem of spurious associations attributable to multiple hypothesis testing. In contrast to candidate gene studies, genomewide association studies use markers that are evenly spaced throughout the genome, without regard to their function or location in a particular gene. Therefore, no previous hypothesis is required. This approach has been shown to be feasible\textsuperscript{31} but poses significant challenges in terms of sample sizes, genotyping resources, and data analysis.
The best way to convincingly demonstrate a true relationship between a genetic marker and disease is replication in independent samples. Yet, many initial findings have not, or cannot, be replicated by subsequent association studies. There are 3 possible explanations for these inconsistencies: false-positive studies, false-negative studies, and true differences between populations.

A major cause for false-positive and false-negative results is inadequate sample size. A recent meta-analysis of 370 association studies found sample size of the initial study was a main predictor of replication failure. This is compounded by publication bias, which remains a major issue. Other important causes for false-positive or false-negative studies include population stratification, misclassification (genotyping or phenotyping errors), and inappropriate statistical methods.

True variability in association between different populations may exist because of differences in: (1) the frequency of disease-causing alleles, (2) the pattern of association between disease-causing alleles and single markers or haplotypes under investigation, or (3) interacting genetic or environmental factors. Thus, nonreplication does not necessarily imply lack of causality but might point to the need for additional studies. In view of these difficulties, it has been proposed recently that replication studies should undertake more extensive genotyping, comprising all genetic variation within a candidate gene. However, it is still unclear how extensively one needs to analyze a gene to exclude association in another population.

In cases of positive replication, the first study to report an association often finds a stronger effect than seen in subsequent studies, particularly if the initial results were obtained through genomewide scans. Therefore, replication studies should be powered to detect effect sizes that are smaller than the initial effect size reported. Meta-analyses may then assist in estimating the population-wide effects of genetic risk factors, although careful account needs to be taken of the effect of publication bias.

Suggestions for Standard Criteria

This discussion has documented the methodological considerations required to increase the likelihood of a valid and replicable study. Major points are listed in Table 2. Few studies will yet meet all criteria; in particular, current sample sizes are smaller than those optimal. We propose standard criteria for implementation in stroke studies to ensure quality and enable replication studies and meta-analyses:

- The a priori hypotheses of the study should be stated upfront.
- Power calculations should be provided to demonstrate that the study is sufficiently powered to test all hypotheses of the study.
- For replication studies, the sample should be large enough to detect or exclude an effect of somewhat smaller size than in the initial study.
- Any overlap with previously published studies (same polymorphisms or polymorphisms in LD with polymorphisms from a previous study in the same sample of patients or controls) should be indicated.

### Data Analysis and Statistical Issues

So far, most association studies have been limited to single polymorphisms (single-nucleotide polymorphisms, repeat polymorphisms, insertions, or deletions). More recently, studies have looked at the joint effects of single markers on a haplotype level. Haplotypes are a set of alleles that are physically close to each other on the DNA strand and are thus inherited as a unit, (ie, a set of alleles that are in strong linkage disequilibrium [LD]). Association between a polymorphism or haplotype and a trait, such as stroke, does not necessarily imply causality. Instead, the polymorphism/haplotype under investigation may be in LD with the causative sequence variant (which may be unknown). In this case, the associated polymorphism or haplotype represents a marker for the causative sequence variant, and more detailed studies are required to identify the causative sequence alteration.

Increasing the number of markers or performing subgroup analyses on stroke subtypes inevitably leads to the problem of multiple testing and the possibility of false-positive associations if correction for multiple testing is not carried out. The loss of statistical power using traditional methods such as the Bonferroni procedure has led many authors to neglect their use. Such methods assume the different comparisons are independent, which is not the case for closely linked polymorphisms; therefore, such methods can be overly conservative and lead to a failure to detect real associations. Alternative algorithms have been proposed that limit the loss of power. When analyzing multiple polymorphisms that are in LD, special algorithms (eg, permutation procedures) should be used.

Because of the paucity of disease loci in the human genome (low prior probability) and the poor track record of candidate gene studies, some geneticists suggest more rigorous significance levels for association studies (eg, \( \alpha=0.005 \) corrected for multiple testing). However, in situations in which the prior probability of association is much higher than average (eg, in the presence of positional data) or in which polymorphisms are linked, such stringency may not be needed. Decision-making in this area is still difficult. For practical purposes, it is recommended that power calculations are performed before starting a study, and adjustment is made if multiple genes are being explored.

### The Issue of Replication

The best way to convincingly demonstrate a true relationship between a genetic marker and disease is replication in...
TABLE 2. Methodological Aspects That Enhance the Significance of an Association Finding

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Requirements</th>
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<tr>
<td>Large sample size</td>
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<td>Rigorous phenotyping in patients and controls</td>
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<td>Low genotyping error rate</td>
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<td>Genomic controls or other techniques to account for population stratification</td>
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<td>Low P value (corrected for multiple testing)</td>
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<td>Odds ratio or attributable risk is high</td>
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<td>Replication in ≥1 independent populations</td>
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<tr>
<td>Gene makes biological sense</td>
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<tr>
<td>Allele affects the gene in a meaningful way*</td>
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<tr>
<td>Presence of a gene–dose response relationship**</td>
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<td>Functional data demonstrating a biological effect of the at-risk allele</td>
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</tbody>
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*It may be difficult to determine whether an allele affects a gene in a meaningful way. If the functional relevance of the polymorphism is unknown, additional experiments examining its biological effect should be considered.

**A description of how cases and controls were sampled should be provided. Controls should be ethnically matched and derived from the same source population as cases. They should allow for the possibility of using other methods to account for population stratification besides matching for ethnicity.

- A description of how cases and controls were sampled should be provided. Controls should be ethnically matched and derived from the same source population as cases. They should allow for the possibility of using other methods to account for population stratification besides matching for ethnicity.

- The genotyping protocol should be specified. In particular, the proportion of patients having CT/MRI, imaging of large extracranial and intracranial arteries (eg, by ultrasound or magnetic resonance angiography), and echocardiography should be detailed.

- Phenotyping of patients should be done according to a standard pathophysiological classification such as the TOAST system and ideally by a central rater particularly for multicenter studies.

- When controlling for conventional risk factors, the definitions and methods used to determine presence or absence of a risk factor should be specified.

- Gene–environment interactions should be sought, but studies need to be powered to allow these analyses.

- Investigators involved in genotyping should be blinded with regard to phenotypes.

- The genotyping error should be stated and low.

- It should be indicated whether marker genotypes were in Hardy–Weinberg equilibrium (HWE) in cases and controls. Strong deviation from HWE (especially in controls) points to the possibility for genotyping errors.

- In an initial association study, data on major stroke subtypes should be presented whenever possible.

- Genotype frequencies as well as allele frequencies should be presented for all groups analyzed.

- The relative risks (odds ratios and 95% CIs) or attributable risks should be indicated.

- Uncorrected P values should be reported to facilitate meta-analyses, but adjustment for multiple testing should still be performed.

- Authors should check for a possible gene–dose effect. If present, the results are usually more convincing. If there is no such effect, it should be discussed whether the mechanism for a dominant or recessive effect is explicable from a biological viewpoint.

- Authors should provide a complete listing of all phenotypes, single markers, and haplotypes analyzed.

- By checking at these criteria during the planning phase of a study, more consistent quality will be obtained.

Conclusion

Association studies remain a powerful tool to identify genetic risk factors for stroke. However, the results from initial reports need to be treated with caution, and replication is essential. Properly conducted negative studies are of interest to the field, especially when addressing previously published claims. To minimize publication bias, journals should be willing to publish high-quality replication studies regardless of whether the results are positive or negative. Efforts should not be wasted on poorly designed studies. Insufficient sample size remains a key issue in most association studies, and collaborative multicenter efforts are needed to solve this problem.

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References


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