Genetic Imbalance Is Associated With Functional Outcome After Ischemic Stroke

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Background and Purpose—We sought to explore the effect of genetic imbalance on functional outcome after ischemic stroke (IS).

Methods—Copy number variation was identified in high-density single-nucleotide polymorphism microarray data of IS patients from the CADISP (Cervical Artery Dissection and Ischemic Stroke Patients) and SiGN (Stroke Genetics Network)/GISCOME (Genetics of Ischaemic Stroke Functional Outcome) networks. genetic imbalance, defined as total number of protein-coding genes affected by copy number variations in an individual, was compared between patients with favorable (modified Rankin Scale score of 0–2) and unfavorable (modified Rankin Scale score of ≥3) outcome after ischemic stroke (IS).

*Dr. Pfeiffer, Chen, and Schlicht contributed equally.
3 months. Subgroup analyses were confined to patients with imbalance affecting ohnologs—a class of dose-sensitive genes, or to those with imbalance not affecting ohnologs. The association of imbalance with outcome was analyzed by logistic regression analysis, adjusted for age, sex, stroke subtype, stroke severity, and ancestry.

**Results**—The study sample comprised 816 CADISP patients (age 44.2±10.3 years) and 2498 SiGN/GISCOME patients (age 67.2±14.2 years). Outcome was unfavorable in 122 CADISP and 889 SiGN/GISCOME patients. Multivariate logistic regression analysis revealed that increased genetic imbalance was associated with less favorable outcome in both samples (CADISP: *P*=0.0007; odds ratio=0.89; 95% CI, 0.82–0.95 and SiGN/GISCOME: *P*=0.0036; odds ratio=0.94; 95% CI, 0.91–0.98). The association was independent of age, sex, stroke severity on admission, stroke subtype, and ancestry. On subgroup analysis, imbalance affecting ohnologs was associated with outcome (CADISP: odds ratio=0.88; 95% CI, 0.80–0.95 and SiGN/GISCOME: odds ratio=0.93; 95% CI, 0.89–0.98) whereas imbalance without ohnologs lacked such an association.

**Conclusions**—Increased genetic imbalance was associated with poorer functional outcome after IS in both study populations. Subgroup analysis revealed that this association was driven by presence of ohnologs in the respective copy number variations, suggesting a causal role of the deleterious effects of genetic imbalance. ([Stroke. 2019;50:298-304. DOI: 10.1161/STROKEAHA.118.021856.]

**Key Words:** DNA copy number variations ■ genetics ■ polymorphism, single nucleotide ■ prognosis ■ stroke
CADISP Study Sample
A total of 983 patients with CeAD diagnosis, based on criteria widely accepted in the stroke community, were included in the CADISP study between 2004 and 2009. In addition, 658 patients with IS attributable to causes other than CeAD (non-CeAD-patients) were enrolled. All patients were self-reported Europeans of white ancestry. DNA was genotyped with Illumina Human 610-Quad or Human 660W-Quad Bead Chips and analyzed in GWAS. Rare CNVs had been analyzed before in 833 CADISP IS patients who were nondisabled before stroke (ie, premorbid modified Rankin Scale [mRS] score of 0) and with microarray data of sufficient quality and complete documentation of sex, age, stroke severity on admission available. Seventeen patients were excluded from the current study because of missing information on outcome. CNV data from the remaining 816 CADISP patients were analyzed about association with functional outcome.

GISCOME Study Sample
The SiGN/GISCOME study was described in detail elsewhere. In short, GISCOME recruited 8831 IS patients with genotype and outcome data to examine the relationship between the 2. For the present study, SiGN/GISCOME individuals were included if they had been genotyped in the SiGN GWAS, using the Illumina Omni 5M genotype platform, and if initial stroke severity information according to National Institutes of Health Stroke Scale and outcome data at 60 to 190 days according to mRS were available. For quality reasons, 165 cases with >20 CNV calls by PennCNV (n=127) and/or mosaicism (n=69) were excluded. Thus, of the 2663 cases with mRS, National Institutes of Health Stroke Scale, and TOAST (Trial of ORG 10172 in Acute Stroke Treatment) stroke sub classification/Cause classification of Stroke system data available, from 6 different study centers, 2498 (93.8%) were included in the final SiGN/GISCOME sample (Table I in the online-only Data Supplement). Excluded and nonexcluded patients did not differ about baseline characteristics, IS subtype, stroke severity on admission, or functional outcome 3 months after stroke (Table II in the online-only Data Supplement).

CNV Analysis
Genetic imbalance was identified as described elsewhere. Briefly, after PennCNV analysis of normalized GWAS microarray data to identify putative CNVs, and after rejection of low-quality results or small candidate variants (comprising <20 SNPs, for 610/660K microarrays and <100 SNPs, for 5M microarrays), all calls were validated by visual inspection after noise reduction (details in the online-only Data Supplement note on CNV validation). CNVs were classified as genic if they comprised the deletion of at least one coding exon, or a duplication that encompassed either an entire coding region or internal exons. In the CADISP population, only rare CNVs were analyzed (ie, <3 findings among the 3703 disease-free subjects in 2 high-quality CNV databases). The CNV findings from the SiGN/GISCOME population were analyzed without frequency filtering. However, for consistency, an additional analysis was performed on rare CNVs only. The genetic imbalance level of an individual was defined as the total number of protein-coding genes affected by CNVs. Finally, we identified all strict ohnologs among the imbalanced genes according to the Ohnologs Browser (http://ohnologs.curie.fr/cgi-bin/BrowsePage.cgi) and categorized imbalance as either including or not-including at least one strict ohnolog.

Baseline Characteristics of Patients
The following clinical variables were included in the analysis: age, sex, ischemic stroke subtype (CADISP: CeAD versus non-CeAD; SiGN/GISCOME: TOAST stroke sub classification subtype, except Lund, Sweden, where IS subtype was determined according to the Causative Classification of Stroke system), and stroke severity on admission (assessed by National Institutes of Health Stroke Scale).

Outcome Evaluation
Functional outcome after 3 to 6 months in the CADISP sample and after 2 to 5 months in the SiGN/GISCOME sample was assessed by the mRS. Outcome was dichotomized for all analyses as favorable (mRS score of 0, 1, or 2) or unfavorable (mRS score of 3, 4, 5, or 6).

Principal Components Analysis
Patients with outlier positions in an ancestry-informative principal components analysis were removed in previous quality control steps. A second principal components analysis was performed on 50000 randomly chosen SNPs to adjust the logistic regression models for ancestry. The 10 major principal components were used as potential confounders.

Statistical Analyses
In both studies, patients with favorable and unfavorable outcome were compared about sex, age, stroke subtype, stroke severity, genetic imbalance level, and country of recruitment by using χ² test, Student t test, or Mann-Whitney U test, as appropriate (univariate analysis). Logistic regression models were used to analyze the association between favorable outcome and genetic imbalance level (multivariate analysis). Results were expressed as odds ratios (OR) per gene affected by a CNV, with 95% CI. In the main analysis, the individual-specific number of protein-coding genes affected by CNVs (ie, the level of genetic imbalance) was included as an independent variable in the model, which was then adjusted for sex, age and the first 10 principal components as possible confounders, and included stroke subtype and stroke severity as additional, potentially relevant covariates. To investigate the impact of genetic imbalance in more detail, we also performed a subgroup analysis distinguishing between patients carrying, or not carrying, at least 1 CNV with an ohnolog. In another subgroup analysis, we compared patients with ohnolog-negative imbalance with the remaining patients.

To explore the validity of the statistical approach taken, and to cover different aspects of the association between outcome and genetic imbalance, we performed additional logistic regression analyses. First, we analyzed the association only between outcome and exceptionally large imbalances, defined as comprising 3 or more protein-coding genes (corresponding to the upper 5% of imbalances observed in our study), as compared with subjects without large imbalances. We also used propensity scores to cover more comprehensively the influence of potential confounders and adjusted the original analysis also for hypertension, diabetes mellitus, and smoking status for CADISP and, additionally, atrial fibrillation for SiGN/GISCOME. In the SiGN/GISCOME population, an analysis with rare variants only was performed as well. Detailed descriptions and summary of the results of the additional analyses are provided in Table III in the online-only Data Supplement. All models were evaluated by pseudo-R² values using the Cox and Snell method. Statistical analyses were performed with R and SPSS 19.0 statistics software packages.

Ethics
The study protocol was approved by relevant local authorities in all participating centers and complied with national regulations concerning ethics committee approval and informed consent.

Results
Figure I in the online-only Data Supplement illustrates the highly dispersed chromosomal localization of CNVs, both for patients with favorable (upper) and unfavorable outcome (lower) in both study populations. Most CNVs were rare. For example, the most frequent recurrent finding (on chromosome 22) was observed in 148 SiGN/GISCOME patients (5.9%). X-chromosomal imbalances were not analyzed in the SiGN/GISCOME population. In the CADISP sample, CNVs had been frequency filtered in an earlier investigation and only
findings with a minor allele frequency ≤0.1% were available for the current study.

The CADISP population included 816 young IS patients (age 44.2±10.3 years). Univariate analyses revealed that patients with unfavorable outcome (n=122) were older than those with favorable outcome (mean age 46.4 versus 43.8 years; P=0.002), less often female (32.8% versus 42.8%, P=0.046), and had more severe strokes (Table 1). The mean genetic imbalance level of patients with unfavorable outcome was larger than for patients with favorable outcome, but the difference was not statistically significant (univariate P=0.24). Logistic regression analysis involving multiple outcome predictors revealed that the negative association between favorable outcome and genetic imbalance level was significant (P=0.001; OR=0.89; 95% CI, 0.82–0.95) and independent of stroke subtype, stroke severity, age, sex, and center of recruitment (Figure; Table 1). Adjustment of the outcome analysis with additional risk factors (hypertension, diabetes mellitus, and smoking status for CADISP and additionally also atrial fibrillation for SiGN/GISCOME) did not significantly affect the observed association between outcome and genetic imbalance (Kruskal-Wallis test; P=0.32). Stroke subtype was not significantly associated with genetic imbalance (Kruskal-Wallis test; P=0.69). When the logistic regression analysis was stratified by TOAST stroke sub classification/Causative Classification of Stroke subtype, imbalance was significantly associated with stroke outcome in the Cardio-Embolic stroke subgroup (n=830) but not in the subgroups with large vessel disease, small vessel disease, or stroke of other or undetermined causes (Table IV in the online-only Data Supplement).

The additional models focusing on large imbalances only (CADISP P=0.001 and SiGN/GISCOME P=0.017) or using propensity scores (CADISP P=0.002 and SiGN/GISCOME P=0.012) also yielded significant associations between imbalance level and outcome in both study groups (Table III in the online-only Data Supplement). An additional analysis with rare variants only, performed in the SiGN/GISCOME population, yielded similar results (OR=0.94; 95% CI, 0.91–0.98; P=0.006).

Table V in the online-only Data Supplement provides an overview of the number of CNVs in patients with respective outcome. No significant associations of individual CNVs with stroke outcome were found after Bonferroni corrections, although 2 CNVs (a duplication in chromosome 7p encompassing the whole Aryl hydrocarbon receptor [AHR] gene and a duplication in chromosome 17 including GPR142, RPL38, TTYH2, DNA12, CD300A, GPRC5C, KIF19, GPR142, BTB17, CD300LB, CD300E, CD300C, and CD300LD) were nominally more common (Fisher exact test P=0.001, without correction for multiple testing) in patients with unfavorable outcome (Figure II in the online-only Data Supplement).

We also assessed the effect of ohnolog-positivity (Figure; Tables 1 and 2). In both cohorts, confining the analysis to ohnolog-positive CNVs replicated the overall imbalance-outcome association (CADISP P=0.002, OR=0.88; 95% CI, 0.80–0.95 and SiGN/GISCOME P=0.002, OR=0.93; 95% CI, 0.89–0.98) whereas analysis of ohnolog-negative CNVs

Table 1. Predictors of Favorable Outcome After Stroke in the CADISP Cohort

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Outcome</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unfavorable (n=122)</td>
<td>Favorable (n=694)</td>
</tr>
<tr>
<td>Female sex (n)*</td>
<td>40 (32.8)</td>
<td>297 (42.8)</td>
</tr>
<tr>
<td>Age (mean±SD)</td>
<td>46.4±8.5</td>
<td>43.8±10.6</td>
</tr>
<tr>
<td>CeAD cause (n)*</td>
<td>70 (57.4)</td>
<td>323 (46.5)</td>
</tr>
<tr>
<td>NIHSS (median)†</td>
<td>14 [0–40]</td>
<td>2 [0–24]</td>
</tr>
<tr>
<td>Imbalance (median/mean)†</td>
<td>0/0.94 [0–29]</td>
<td>0/0.51 [0–28]</td>
</tr>
<tr>
<td>Imbalance with ohnologs (median/mean)†</td>
<td>0/0.57 [0–28]</td>
<td>0/0.23 [0–28]</td>
</tr>
<tr>
<td>Imbalance without ohnologs (median/mean)†</td>
<td>0/0.30 [0–8]</td>
<td>0/0.25 [0–25]</td>
</tr>
</tbody>
</table>

The association between functional outcome and different types of genetic imbalance was assessed by multivariate logistic regression analysis (model 1: continuous genetic imbalance), each time adjusted for age, sex, and ancestry-derived principal components 1–10 as potential confounders, and including stroke subtype (CeAD vs non-CeAD) and stroke severity (NIHSS) as additional covariates. Univariate P values obtained by nonmodel-based methods: χ² test, Student t test, or Mann-Whitney U test, as appropriate. CADISP indicates Cervical Artery Dissections and Ischemic Stroke Patients; CeAD, cervical artery dissection; NIHSS, National Institutes of Health Stroke Scale; OR, model-adjusted odds ratio of favorable outcome.

*Percentage in brackets.
†Range in square brackets.
The present exploratory study of genetic imbalance and stroke outcome in patients with IS yielded the following key findings: (1) the risk of unfavorable outcome increased with the number of protein-coding genes involved in genetic imbalance; (2) the observed association between genetic imbalance and outcome was independent of age, sex, stroke subtype, and stroke severity; and (3) the association between outcome and ohnolog-positive, putatively pathogenic imbalance was statistically significant, whereas that with ohnolog-negative imbalance was not.

Discussion

The choice of microarray platform, CNV detection algorithm, filtering strategy, and CNV validation method may all affect the results of genetic imbalance studies.\textsuperscript{8,19,20} We previously tested different algorithms for CNV detection, including PennCNV, QuantiSNP, Birdsuite, and software from the Affymetrix Genotyping Console.\textsuperscript{5} In our clinical DNA samples of variable quality, all detection algorithms were underperforming, which motivated us to systematically study false CNV detection and noise in SNP microarray data and develop new protocols for high throughput CNV validation after noise reduction.\textsuperscript{17} Extensive manual curation of CNV findings was performed in the present study, even after the exclusion of low-quality samples. Many CNV findings were recurrent and strongly overlapped with CNVs reported in public databases. Moreover, some of the CNV findings from the CADISP samples have been validated elsewhere, using independent molecular methods, including quantitative polymerase chain reaction and sequencing of breakpoint-joining polymerase chain reaction fragments.\textsuperscript{6}

The above notwithstanding, CNV detection in SNP microarray data is challenging because these platforms were not primarily designed for CNV detection. Also, regions with segmental duplications are rich in CNV, but these regions are underrepresented in microarray platforms and hard to cover by next generation sequencing. These shortcomings may explain why some common CNVs with potential impact on IS\textsuperscript{21,22} were not identified in the current study. Moreover, SNPs within regions of segmental duplication or common CNVs are likely to violate Hardy Weinberg equilibrium\textsuperscript{23,24} and may, therefore, be rare among microarray platform probe sets.

Information on premorbid mRS, cardiovascular risk factors, and complications during acute hospitalization and early follow-up in the CADISP and SiGN/GISCOME studies were incomplete, which is a limitation of our study. Furthermore, we were unable to analyze mRS on a continuous scale since this information was missing for some centers. Stratification by type of unfavorable outcome, for

Table 2. Predictors of Favorable Outcome After Stroke in the SiGN/GISCOME Cohort

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Outcome</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfavorable (n=889)</td>
<td>Favorable (n=1609)</td>
</tr>
<tr>
<td>Female sex (n)*</td>
<td>459 (51.6)</td>
<td>580 (36.0)</td>
</tr>
<tr>
<td>Age (mean±SD)</td>
<td>73.7±12.9</td>
<td>64.3±13.7</td>
</tr>
<tr>
<td>TOAST/CCS subtype</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NIHSS (median)†</td>
<td>7 [0–41]</td>
<td>3 [0–30]</td>
</tr>
<tr>
<td>Imbalance (median/mean)†</td>
<td>0/0.18 [0–48]</td>
<td>0/0.90 [0–27]</td>
</tr>
<tr>
<td>Imbalance with ohnologs (median/mean)†</td>
<td>0/0.69 [0–48]</td>
<td>0/0.37 [0–27]</td>
</tr>
<tr>
<td>Imbalance without ohnologs (median/mean)†</td>
<td>0/0.49 [0–14]</td>
<td>0/0.53 [0–13]</td>
</tr>
</tbody>
</table>

The association between functional outcome and different types of genetic imbalance was assessed by multivariate logistic regression analysis (model 1: continuous genetic imbalance), adjusted for age, sex, and ancestry-derived principal components 1–10 as potential confounders, and including stroke subtype (TOAST/CCS) and stroke severity (NIHSS) as additional covariates. Univariate P values obtained by nonmodel-based methods: \(\chi^2\) test, Student t test, or Mann-Whitney U test, as appropriate. CCS indicates Causative Classification System; GISCOME, Genetics of Ischaemic Stroke Functional Outcome; n.d., not done; NIHSS, National Institutes of Health Stroke Scale; OR, model-adjusted odds ratio of favorable outcome; SiGN, Stroke Genetics Network; and TOAST, Trial of ORG 10172 in Acute Stroke Treatment stroke sub classification.

*Percentage in brackets.
†Range in square brackets.
example, mRS, National Institutes of Health Stroke Scale, or cognitive function, may improve the analysis of outcome predictors further, including genetic factors. Another possible drawback of our study may be that the 2 samples of IS patients differed with regard to some important aspects: The CADISP study population was younger, on average, than the SiGN/GISCOME population, which matters because age itself is a predictor of outcome. Nevertheless, genetic imbalance level remained associated with outcome in both study samples after adjustment for age.

The ischemic stroke subtypes of the 2 cohorts in our study were also notably different which may at least partially explain why the results in the 2 cohorts were not identical. A stronger association between outcome and genetic imbalance was seen in the CADISP cohort, and a subgroup analysis in SiGN/GISCOME revealed that the association was confined to patients with cardio-embolic stroke, an IS subgroup that was not specifically analyzed in CADISP. This notwithstanding, it cannot be excluded that CNV burden is also a more general determinant of human physiology, a concept supported by previous reports of a relationship with for example, common variable immunodeficiency,25 neuropsychiatric,26 or other anthropometric traits.27 Thus, general mechanisms of recovery that help to avoid complications of stroke may be impaired in subjects with higher genetic imbalance, independent of individual stroke subtype.

Imbalance identification was performed using different microarray platforms, which resulted in different cutoffs for filtering of the CNV findings. By including only CNVs of >20 SNPs in CADISP and >100 SNPs in SiGN/GISCOME, we may have missed smaller variants, implying that we might even have underestimated the influence of genetic imbalance. Finally, for the CADISP sample, only rare CNVs were available whereas, in the SiGN/GISCOME population, we performed ab initio CNV identification without frequency filtering. However, an additional analysis of the SiGN/GISCOME data with frequency filtering for rare variants hardly changed the association observed between imbalance level and outcome.

Strengths of our study include well-characterized, large study samples and a consistent association between genetic imbalance level and outcome after IS observed in 2 independent study samples. Adjustment for age, sex, stroke severity, and ancestry suggests independence of these associations from potential confounders. In addition, our key finding was corroborated by subgroup analyses of ohnolog-positive and ohnolog-negative genetic imbalance. Ohnologs are likely to be dosage-sensitive genes as they are refractory to CNV and have rarely experienced small-scale duplication.28 Consistent with this, ohnologs have been associated with disease and to be overrepresented in pathogenic CNVs.11 Comparing ohnolog-positive and ohnolog-negative imbalances may further identify genes driving the pathogenicity of CNVs. Our finding that imbalance enriched in ohnologs is associated with worse outcome is in line with the hypothesis that dosage-sensitive ohnologs play a role in the pathogenicity of CNVs. Importantly, the high quality of CNV validation in the current study should have served to minimize the rate of false-positive CNV detection and, thus, to improve the reliability of the results. In fact, visual CNV inspection is a valuable complement to common CNV calling algorithms.

Our study also tried to capture the complex architecture of disease outcome. Common SNPs account only for a small percentage of phenotypic variation, and by investigating genetic imbalance, we added structural variants to the repertoire of potential predictors of outcome after IS, which may help to detect some of the missing heritability and be diagnostically useful.29 A general concept of genetic imbalance, rather than imbalance of a specific genetic locus, was preferred in our study because of the complexity of the outcome phenotype after IS.

Further studies are needed to evaluate the causes of the identified associations. The notion of genetic imbalance is fairly unspecific and indicates the loss or gain of any protein-coding gene. Future research should examine if stroke outcome is related to imbalance in specific, predefined sets of genes, for example, in genes associated with biological processes like inflammatory response or specific pathways like TGF-β (transforming growth factor β) receptor signaling. One theme could be if genetic imbalance causes a general loss of resilience and thus affects stroke recovery rates and susceptibility to other diseases. Another idea would be to focus on specific genes involved in disease-relevant pathways, such as inflammation, cell death, or neuronal recovery/plasticity. Future pathway-based CNV investigations might lead to identification of gene families relevant to outcome after IS and provide further insight into the mechanisms of stroke recovery.

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Disclosures

Dr Lindgren reports speech and seminar honoraria from Bayer and BMS/Pfizer and consulting for Bayer, AstraZeneca, Boehringer Ingelheim, BMS/Pfizer and Reneuron. The other authors report no conflicts.

References


