PAPER

Evaluation of the modifying effects of unfavourable genotypes on classical clinical risk factors for ischaemic stroke

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Objectives: Ischaemic stroke is a frequent heterogeneous multifactorial disease that is affected by a number of genetic mutations and environmental factors. We hypothesised the clinical importance of the interactions between common, unfavourable genetic mutations and clinical risk factors in the development of ischaemic stroke.

Methods: The Factor V Leiden G1691A (Leiden V), the prothrombin G20210A, the methylenetetrahydrofolate reductase C677T (MTHFR C677T) mutations, the angiotensin converting enzyme I/D (ACE I/D), and apolipoprotein allele e4 (APO e4) genotypes were examined by the polymerase chain reaction (PCR) technique in 867 ischaemic stroke patients and 743 healthy controls. Logistic regression models were used to estimate the roles of the co-occurrences of the clinical risk factors and common genetic mutations in ischaemic stroke.

Results: The Leiden V mutation in combination with hypertension or diabetes mellitus increased the risk of ischaemic stroke. We found synergistic effects between the ACE D/D and MTHFR 677TT genotypes and drinking or smoking. The presence of the APO e4 greatly facilitated the unfavourable effects of hypertension, diabetes mellitus, smoking, or drinking on the incidence of ischaemic stroke.

Conclusion: In certain combinations, pairing of common unfavourable genetic factors, which alone confer only minor or non-significant risk, with clinical risk factors can greatly increase the susceptibility to ischaemic stroke.

Ischaemic stroke is a common multifactorial disease; several genetic and environmental factors together can lead to its development. A number of frequent polymorphisms and mutations have been proposed as genetic risk factors.[1 2] Although some of them can give rise to a specific stroke subtype as minor but significant risk factors,[3] most of them alone have not proved to be significant risk factors for ischaemic stroke overall. It has been demonstrated that a clustering of unfavourable mutations can give rise to stroke or to other circulatory disorders—for example, leukoaraiosis—even if alone they have not proved to be significant risk factors.[4 6] In spite of the real possibility of interactions of the genetic and clinical risk factors, only a few such data are available in the current literature of stroke research.[7 8] The Factor V Leiden G1691A (Leiden V),[9–29] the angiotensin converting enzyme D allele (ACE D),[30–36] the methylenetetrahydrofolate reductase C677T (MTHFR C677T),[37–40] the prothrombin G20210A[41–42] mutations, and the apolipoprotein allele e4 (APO e4)[43–47] are considered unfavourable minor or non-significant genetic risk factors for ischaemic stroke overall. In this current study, we have examined whether these genetic factors can exert measurable modifying effects on the clinical risk factors and can therefore increase the relative risk of an ischaemic stroke event.

MATERIAL AND METHODS

Study population

The data on 867 consecutive Caucasian Hungarian patients with acutely developing ischaemic stroke, who had never suffered a previous stroke event, were analysed. These subjects had been admitted to the Department of Neurology and Neurophysiology at Pándy Kálmán County Hospital between January 1 1998 and June 25 2002, after being examined by a physician in the emergency admissions ward or by a general practitioner at their homes. All 867 subjects underwent a detailed clinical scrutiny, including an exploration of the medical history and the family anamnesis, an evaluation of the vascular risk factors, general physical and neurological examinations, urine analysis, extended laboratory examinations, electrocardiography, extracranial and transcranial Doppler sonography of the carotid and cerebral arteries, transthoracic and/or transoesophageal echocardiography where appropriate, and magnetic resonance imaging (MRI) examinations, within 2 days of the first observation of symptoms. The MRIs were produced with a 0.23 T resistive magnet (Picker, Outlook, Finland), using a head coil. The infarctions were evaluated in the axial and coronal views of the T2, T1, and proton density weighted images. All scans were read by an experienced investigator blinded to the clinical and laboratory data.

The study design was prospective; following evaluation of the radiological features, the patients were enrolled into three subgroups. Group 1 represented small vessel occlusion (one or more subcortical hemispheric or brainstem infarcts with a diameter less than 1.5 cm on MRI); group 2 represented large vessel infarction (cortical or cerebellar lesions and/or brainstem infarcts, or subcortical hemispheric infarcts, greater than 1.5 cm in diameter on MRI); group 3 represented a mixed vascular pathology (one or more lacunar or large vessel infarcts on MRI). Subjects whose MRIs could not be

Abbreviations: ACE, angiotensin converting enzyme; PCR, polymerase chain reaction; MRI, magnetic resonance imaging; CT, computerised tomography; TIA, transient ischaemic attack; EGC, electrocardiogram; BMI, body mass index; EDTA, ethylene diamine tetra-acetic acid; OR, odds ratio; CI, confidence interval

recorded, or whose clinical parameters and risk factors were uncertain, were excluded.

As a control group, 743 healthy Caucasian Hungarian individuals were examined, having been randomly selected using age and sex matching from general practice registers from the same locality as the cases, with the requirement that their brain MR or CT (computerised tomography) imaging showed no evidence of stroke or other neurological or cerebrovascular abnormality. Subjects with any kind of previous clinical data suggesting a cerebrovascular or cardiovascular event, such as a transient ischaemic attack (TIA) or angina pectoris, were excluded from the control group. The frequencies of mutations in our control group were similar to those found in control groups in other genetic studies.[1 2 6] The frequencies of the examined combination pairs of genotypes and clinical risk factors in our control group accorded with statistically expectable probability. This distribution of genotypes among a random selection from a large population medically indifferent to the examined genotypes could exclude selection bias, which might have affected the distribution of genotypes in the control group. Both the controls and the patients gave their informed consent to the clinical work up and the DNA analysis.

Assessment of clinical data

Smoking and drinking habits and the presence of hypertension or diabetes mellitus were recorded for all participants. The serum cholesterol level, serum triglyceride level, platelet count, and haematocrit were also measured and analysed as important clinical parameters. Hypertension was diagnosed when the blood pressure repeatedly exceeded 160 mm Hg systolic and/or 95 mm Hg diastolic when the subject was taking antihypertensive medication. Diabetes mellitus was diagnosed when the glucose level was at least 7.78 mmol/l in a fasting state, and/or at least 11.11 mmol/l two hours after a meal or after 75 g oral glucose, according to the World Health Organization criteria.[48] The data on hypertension and diabetes mellitus were obtained from the medical histories or at least two weeks after the stroke event. Ischaemic heart disease was diagnosed on a history of angina pectoris or acute myocardial infarction or if electrocardiograph (ECG) showed evidence of coronary heart disease.

Participants were classified as smokers if they had ever smoked more than five cigarettes per day for at least a year, and as moderately heavy drinkers if they drank 5 units of alcohol or more per day.

DNA analysis

Leiden V, prothrombin G20210A and MTHFR C677T mutations, ACE I/D polymorphism, and APOE genotypes were examined using polymerase chain reaction (PCR), by operators blinded to the results of the clinical workup. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Kasztel-Med, Budapest), according to the manufacturer's instructions, from 200 µl of peripheral blood anticoagulated with ethylene diamine tetra-acetic acid (EDTA). The conventional PCR technique was used for the Leiden V,[49] the MTHFR C677T,[50] the prothrombin G20210A mutations,[51] and the APO e4 polymorphism.[52] The ACE polymorphism was examined by a PCR method developed to decrease the examination time and provide a better detection of heterozygotes.[53] This method consisted of a fluorescent probe melting point analysis performed with fluorescently labelled oligonucleotide hybridisation probes on the LightCycler™ instrument (Roche Diagnostics, Roche Kft, Budapest).

Statistical analysis

The clinical data were expressed as means (SD) where appropriate. The differences between the clinical parameters, allele frequencies, and genotype frequencies in the stroke group and the controls were assessed using the χ^2 test or the Mann–Whitney test where appropriate. Several logistic regression models were evolved to evaluate the importance of the co-occurrences of the different genotypes and the significant clinical risk factors in the development of ischaemic stroke. In the models, we calculated odds ratios (ORs) for each significant clinical risk factor and then, step by step, for every possible combination of pairs of the main unfavourable genotypes and significant clinical risk factors, after adjustment for differences in age and all the remaining significant clinical risk factors. Likelihood ratio tests were performed in order to assess the models against each other. The main genotype categories examined were the Leiden V, MTHFR 677T, ACE D, and APO e4 (with scores of 1 for both the homozygous and heterozygous states, and 0 for lack of the given allele); and homozygous MTHFR 677TT and ACE D/D genotypes (with scores of 1 for the homozygous states, and 0 for the heterozygous state and lack of the given allele). We also constructed models in which the effects of the MTHFR 677T and ACE D alleles were assumed to be dose dependent (with scores of 1 for the heterozygous and 2 for the homozygous states, and 0 for lack of the allele).

As a consequence of the great number of statistical comparisons, there arose a chance of statistically significant associations being registered between ischaemic stroke and a given combination pair of the genotypes and significant clinical risk factors by accident, without valid biological associations. In order to decrease this possibility, we randomly divided our stroke group into two samples of identical size, and carried out logistic regression calculations independently for them. We considered an association between ischaemic stroke and the co-occurrence of a given genotype and a clinical risk factor biologically valid if that association was calculated to be statistically significant ($p<0.05$) in both independent stroke samples. Afterwards, the frequencies of these combination pairs only were examined statistically in the undivided original stroke group and in different stroke subtypes. This methodical approach could resolve the statistical problems which stem from exploratory multiple testing. For all the ORs, the 95% confidence intervals (95% CI) were calculated. Logistic regression analyses were performed with the statistical package SYSTAT 10 for Windows.

In order to examine whether the positive significant interactions resulted from positive enhancing effects between the given clinical and genetic risk factors, we divided the case group into two subgroups according to the presence or lack of the first factor (in the given genetic risk factor combination pair). The relative frequencies of the second factor (in the same risk factor combination) in these two groups were compared in a two by two table by the χ^2 test.

RESULTS

The clinical characteristics and genotype distributions of the patients and the control subjects are listed in tables 1 and 2. The mean BMI, serum cholesterol level, and serum triglyceride level were all significantly higher in the stroke group than in the control group. Hypertension, diabetes mellitus, ischaemic heart disease, smoking, and drinking occurred more frequently among the stroke patients than the controls.

The frequency of the APO e4 was significantly higher in the stroke patients (0.25%; $p<0.0005$; OR 2.3, 95% CI 1.3 to 3.6) than in the control group (0.13). No other genotype was associated with a significantly increased risk of ischaemic stroke.

The results from the logistic regression models are given in table 3. Briefly, the Leiden V mutation increased the relative risk of an ischaemic stroke event when it occurred in

Table 1 Characteristics of patients and control subjects

Characteristics	Stroke group	Control group
Total number	867	743
Gender, females/males	411/456	346/397
Age, years	61.2 (15.2)	60.4 (12.2)
BMI, kg/m^2	26.9 (2.0)***	24.1 (2.1)
Cholesterol, mmol/l	6.6 (1.5)***	5.3 (1.3)
Triglycerides, mmol/l	1.82 (0.9)***	1.35 (0.8)
Haematocrit, %	46 (9)	45 (7)
Platelet count, x 10^9	251 (50)	252 (46)
With hypertension	51.0%‡	17.9%
With diabetes mellitus	31.95%‡	5.92%
Smokers	32.99%‡	10.9%
Drinkers	12.92%‡	4.4%
With ischaemic heart disease	14.4%**	8.75%

**p<0.01; *** p<0.001; ‡p<0.0005.
BMI, body mass index.

Table 2 Distribution of the different genotypes among the stroke patients and the control group

Genotypes	Stroke group (n = 867)	Control group (n = 743)
Heterozygous	5	4
Prothrombin G20210A	0.58%	0.5%
Heterozygous Leiden V	69(8%)	47(6.3%)
Homozygous Leiden V	3(0.4%)	2(0.3%)
Homozygous+heterozygous Leiden V	72(8.3%)	49(6.6%)
Leiden V allele frequency	0.04	0.03
Apolipoprotein E 2 allele frequency	0.07	0.09
Apolipoprotein E 3 allele frequency	0.68	0.78
Apolipoprotein E 4 allele frequency	0.25‡	0.13
Homozygous MTHFR C677T	114(13.1%)	89(12%)
Heterozygous MTHFR C677T	338(39%)	268(36%)
ACE I/D	426(49.1%)	373(50.2%)
ACE I/I	208(24%)	200(26.9%)
ACE D/D	233(26.9%)	170(22.9%)
D allele frequency	0.51	0.48
I allele frequency	0.49	0.52

‡ p<0.0005.
n, number.

Table 3 Measurable synergistic effects between unfavourable genotypes and clinical risk factors on the incidence of ischaemic stroke

Clinical risk factors alone or in combination with synergistic unfavourable genotypes	OR (95% CI)
Hypertension	3.6 (6.3 to 1.8)§
Hypertension+Leiden V allele	10.8 (33.1 to 3.5)†
Hypertension+APOE 4 allele	15.6 (52.2 to 3.8)†
Diabetes mellitus	4.1 (8.6 to 2.1)†
Diabetes mellitus+Leiden V allele	8.3 (19.6 to 3.2)†
Diabetes mellitus+APOE 4 allele	18.8 (62.6 to 4.4)†
Smoking	2.3 (4 to 1.4)***
Smoking+ACE D/D	8.5 (19.8 to 3.4)†
Smoking+MTHFR677TT	6.3 (13.7 to 2.8)†
Smoking+APOE 4 allele	16.4 (54.4 to 4.1)†
Drinking	2.9 (5.4 to 1.4)†
Drinking+ACE D/D	12.2 (36.4 to 3.7)†
Drinking+MTHFR 677TT	9.1 (25.2 to 3.4)†
Drinking+APOE allele	11.8 (36.2 to 3.5)†

OR, odds ratios; CI, confidence interval.
§p<0.003; ***p<0.001; †p<0.0001.

combination with hypertension or diabetes mellitus. The ACE D/D and the homozygous MTHFR 677TT mutations exerted synergistic effects in combination with drinking or smoking in the development of ischaemic stroke. The presence of the APO e4 increased the unfavourable influences of hypertension, diabetes mellitus, smoking, and drinking on the incidence of ischaemic stroke. The log likelihood goodness was significantly higher in the models with the synergistic interactions above than in those without them. No other pairs of the main unfavourable genotypes and clinical risk factors yielded a significantly increased risk of ischaemic stroke.

The positive interactions are analysed in tables 4–7. We found that the positive interactions between the various clinical risk factors and the genetic factors resulted from their mutually enhancing effects. Their co-occurrence in the stroke group was significantly higher than was to be expected from their separate occurrences in the stroke group.

The distributions of synergistic interactions among the different stroke subtypes are given in table 8.

DISCUSSION

The distribution of genotypes met the requirements of the Hardy–Weinberg equilibrium. The clinical characteristics among the stroke patients were consistent with earlier data in that an elevated serum cholesterol or serum triglyceride level, smoking, drinking, hypertension, diabetes mellitus, and ischaemic heart disease proved to be clinical risk factors for ischaemic stroke.

Most of the unfavourable genotypes alone did not indicate a significant risk of ischaemic stroke; only the APO e4 was found to be a minor, but significant, risk factor. However, we found that different unfavourable genotypes were able to

Table 4 The relative frequencies of hypertension, diabetes mellitus, smoking, and drinking in cases of ischaemic stroke with and without the APO e4 allele

	Ischaemic stroke cases with APO e4 allele n=358 (%)	Ischaemic stroke cases without APO e4 allele n=509 (%)
With hypertension	231 (64.5)	211 (41.4)
Without hypertension	127 (35.5)	298 (58.6)
With diabetes mellitus	146 (40.8)	131 (25.7)
Without diabetes mellitus	212 (59.2)	378 (74.3)
With smoking	158 (44.1)	128 (25.2)
Without smoking	200 (55.9%)	381 (74.8%)
With drinking	68 (19)	44 (8.6)
Without drinking	290 (81)	467 (91.4)

All p<0.0005.
APO e4, apolipoprotein allele e4.

Table 5 Relative frequencies of hypertension and diabetes mellitus in cases of ischaemic stroke with and without the Leiden V mutation

	Ischaemic stroke cases without Leiden V n=795 (%)	Ischaemic stroke cases with Leiden V n=72 (%)
With hypertension	394 (49.6)	48 (66.7)
Without hypertension	401 (50.4)	24 (33.3)
With diabetes mellitus	240 (30.2)	37 (51.4)
Without diabetes mellitus	555 (69.8)	35 (48.6)

All p<0.0005.
The groups with or without the Leiden V mutation were compared by the χ^2 test.

Table 6 Relative frequencies of smoking and drinking in cases of ischaemic stroke with and without the MTHFR 677TT genotype

	Ischaemic stroke cases without MTHFR 677TT n = 753 (%)	Ischaemic stroke cases with MTHFR 677TT n = 114
With smoking	230 (30.5%)	56 (49.1)
Without smoking	523 (69.5)	58 (50.9)
With drinking	81 (10.8)	31 (27.2)
Without drinking	672 (89.2)	83 (72.8)

All $p<0.005$.
The groups with or without the MTHFR 677TT genotype were compared by the χ^2 test.

Table 7 Relative frequencies of smoking and drinking in cases of ischaemic stroke with and without the ACE D/D genotype

	Ischaemic stroke cases without ACE D/D n = 634 (%)	Ischaemic stroke cases with ACE D/D n = 233 (%)
With smoking	144 (22.7)	142 (60.9)
Without smoking	490 (77.3)	91 (39.1)
With drinking	60 (9.5)	52 (22.3)
Without drinking	574 (90.5)	181 (77.7)

All $p<0.005$.
The groups with or without the ACE D/D genotype were compared by the χ^2 test.

increase the relative risk of an ischaemic stroke event when they occurred in combination with the common clinical risk factors. The Leiden V mutation in combination with hypertension or diabetes mellitus increased the relative risk of ischaemic stroke. The homozygous MTHFR 677TT and ACE D/D genotypes facilitated the unfavourable effects of smoking or drinking on the incidence of ischaemic stroke. Whereas the Leiden V, the homozygous MTHFR C677T, and the ACE D/D genotypes exhibited synergistic effects with only two clinical risk factors, the APO e4 yielded a more general synergistic effect with more clinical risk factors. The presence of at least one APO e4 in combination with hypertension, diabetes mellitus, smoking, or drinking multiplied the relative risk of ischaemic stroke. No synergistic effects were found between the BMI, the serum cholesterol level, the serum triglyceride level, ischaemic heart disease and the unfavourable genotypes.

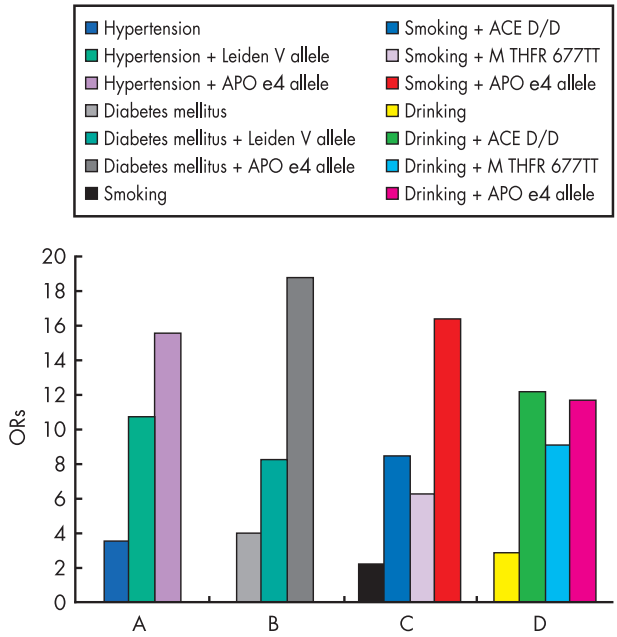

Figure 1 Synergistic effects between unfavourable genotypes and clinical risk factors on the incidence of ischaemic stroke, as significant odds ratios (OR)s, with 95% confidence intervals. A–D show different combinations. APO e4, apolipoprotein allele e4.

The Leiden V mutation in combination with hypertension or diabetes mellitus increased the frequency of large vessel infarcts. The MTHFR 677TT or ACE D/D genotypes combined with smoking or drinking contributed significantly to the development of small vessel infarcts. The APO e4 gave rise significantly to both large vessel and small vessel infarcts.

Despite the large number of statistical comparisons, the high levels of significance of the associations and the statistical approach used (analyses of two randomly evolved independent samples of ischaemic stroke for the final selection of the important and valid combination pairs) strongly decreased the possibility that the associations found might be only of statistical origin.

Our findings suggest some general rules for the interactions of the examined common genetic mutations and common clinical risk factors at the phenotype level. Genetic factors that are minor or insignificant when present alone can not only exert an additive effect, but also facilitate the effects of other common clinical risk factors at a clinical

Table 8 Synergistic effects between unfavourable genotypes and clinical risk factors on the incidence of ischaemic stroke subtypes, as significant ORs (95% CI)

	Subtype of ischaemic stroke		
Risk factors and genotypes	Large vessel	Small vessel	Mixed type
Total number of cases	350	239	278
OR for hypertension+Leiden V	7.3 (27.3 to 2.3)*	NS	13(39.1 to 2.6)*
OR for hypertension + APOE 4 allele	14.3 (61.3 to 3.1)*	13.2 (58.3 to 2.9) *	18(64.3 to 3.4)*
OR for diabetes mellitus + Leiden V	12.3 (45.4 to 2.6)*	NS|	10.3(35.4 to 2.8)*
OR for diabetes mellitus + APOE 4 allele	17.3 (71.3 to 4.1)*	19.3 (72.3 to 3.9)*	18.4(71.3 to 3.8)*
OR for smoking + ACE D/D	NS	11.6 (25.3 to 2.9)*	13.2(28.3 to 2.8)*
OR for smoking + MTHFR 677TT	NS	8.3 (19.6 to 2.1)*	7.2(18.7 to 2.2)*
OR for smoking + APOE 4 allele	16.8 (62.3 to 3.8)*	17.3 (71.3 to 3.3)*	14.2(58.3 to 4.0)*
OR for drinking + ACE D/D	NS	14.2 (41.6 to 3.2)*	13.2(44.3 to 3.1)*
OR for drinking + MTHFR 677TT	NS	12.3 (33.4 to 3.1)*	10.3(29.3 to 2.7)*
OR for drinking + APOE 4 allele	12.6 (42.6 to 2.6)*	11.2 (41.3 to 2.5)*	8.6(29.3 to 2.2)*

OR, odds ratio; CI, confidence interval; NS, not significant.
*$p<0.0001$

phenotype level. Their co-occurrence in the same subject can, therefore, give rise to a highly significant relative risk of an ischaemic stroke event (fig 1). Certain specific pairs of mutations and clinical risk factors in combination can yield highly significant complex risk vectors for ischaemic stroke, illustrated in fig 1. Certain classical clinical risk factors indicate a much higher risk for those patients who are carriers for certain common unfavourable genetic mutations, as shown in fig 1.

CONCLUSION

Besides the classical clinical risk factors, rapidly increasing knowledge of unfavourable genetic mutations will permit recognition of a larger population at high risk of ischaemic stroke, and this may lead to more effective prevention. Our results may be useful in everyday medical practice, in that the detection of some of the common unfavourable mutations, in combination with certain clinical risk factors, in one subject, could be alarming and call attention to the need for much stricter prevention measures. A better understanding of the interactions between genotypes and clinical risk factors may facilitate appropriate and rapid mutation detection.

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