SHORT REPORT

IL1RN intron 2 polymorphism caused by variable number tandem repeats is associated with 1-year outcome in patients with ischaemic stroke

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Background: Results of experimental and clinical studies suggest that recombinant human interleukin 1 receptor antagonist (rhIL1ra) may be a good new therapeutic agent for acute stroke. In humans, IL1ra is encoded by the *IL1RN* gene located on chromosome 2.

Objectives: To report on the association between different genotypes of the variable number tandem repeat (VNTR) polymorphism within *IL1RN*, and disease severity and 1-year outcome in patients with ischaemic stroke.

Methods: *IL1RN* genotypes were evaluated using polymerase chain reaction in 391 patients with ischaemic stroke diagnosed according to the World Health Organization definition. The effects of *IL1RN* genotypes on severity of stroke at maximum impairment, and on the survival status and neurological and functional condition of patients at 7 days, 1 month, 3 months and 1 year after the onset, were evaluated.

Results: No relationship was found between *IL1RN* genotypes and severity of symptoms at the time of maximum impairment. Homozygotes for the *IL1RN**2 allele showed less severe neurological and functional impairments when assessed after the time period between 7 days and 1 year after stroke compared with carriers of the other two *IL1RN* genotypes. Patients with at least one copy of the *IL1RN**2 allele had increased risk of death during the first week, and patients homozygotic for this allele had increased risk of death within the first month after stroke.

Conclusion: *IL1RN* intron 2 variable number tandem repeats polymorphism influences the clinical outcome in patients with ischaemic stroke. It may possibly modify effects of treatment with rhIL1ra in patients with acute stroke.

Over the past few years, a body of evidence has emphasised the substantial role of post-stroke inflammatory response in the progression of ischaemia-induced brain injury.[1 2] In many human studies, the magnitude of the inflammatory response was of prognostic significance in ischaemic stroke.[3–5] Most inflammatory reactions are mediated by cytokines. One of the most powerful proinflammatory cytokines is interleukin (IL)1. The IL1 family consists of IL1α, IL1β, and IL1 receptor antagonist (IL1ra) (http://www.cope-withcytokines.de). IL1ra antagonises signalling by IL1α and IL1β.

Both IL1 agonists and IL1ra are produced rapidly in brains of rodents exposed to cerebral ischaemia.[6 7] Intraventricular IL1-β injection dramatically exacerbated brain damage induced by ischaemia. Adenoviral vector-induced overexpression of IL1ra and also intracerebroventricular or peripheral administration of IL1ra markedly reduced infarct volume and oedema, decreased neuronal loss, decreased glial activation and brain infiltration with peripheral blood cells, and also improved behavioural outcome in animals subjected to middle cerebral artery occlusion. In 2005, results of the first randomised clinical trial were published, suggesting that rhIL1ra may be a safe and well-tolerated new agent for treatment of acute ischaemic stroke.[8]

In humans, IL1ra is encoded by the *IL1RN* gene located on chromosome 2 (2q13; OMIM 147679). The penta-allelic polymorphism caused by variable numbers of an 86-bp tandem repeat (VNTR) sequence has been described in *IL1RN* intron 2. This polymorphism has been shown to influence IL1ra protein production by cells.[9 10]

In previous studies, the *IL1RN* VNTR polymorphism was implicated in susceptibility to ischaemic stroke.[11 12] In this study, we assessed whether carriership of different genotypes of *IL1RN* is associated with severity of disease and with 1-year outcome in patients with ischaemic stroke.

METHODS

The study cohort consisted of 391 patients with ischaemic stroke consecutively admitted to the Stroke Unit, Second Department of Neurology, Institute of Psychiatry and Neurology, Warsaw, Poland. All participants were of the same ethnic origin. Ischaemic stroke was diagnosed according to the World Health Organization definition,[13] supplemented by brain imaging to exclude intracranial haemorrhage or a non-vascular cause. Information about risk factors for stroke and clinical data were collected according to the Stroke Data Bank, National Institutes of Health protocol,[14] including data on age, hypertension (previous diagnosis or values ⩾140 mm Hg (systolic) or ⩾90 mm Hg (diastolic) on at least two subsequent measurements in the post-acute phase of the disease), diabetes (previous diagnosis or a fasting plasma glucose concentration of >7.8 mmol/l), atrial fibrillation (supported by electrocardiogram), smoking (current or former), pathological alcohol drinking (>1000 g a week) and congestive heart failure, and a history of transient ischaemic attack, stroke or myocardial infarction. Presumed stroke aetiology was defined according to the TOAST criteria.[15] Data on aphasia, dysarthria, dysphagia and consciousness level were collected at the time of maximum impairment within the first 7 days after the ischaemic stroke. Neurological impairment was evaluated using the Scandinavian Stroke Scale (SSS)[16] on admission and 7 days and 1 month after the ischaemic stroke. Activities of daily living were measured by the Barthel Scale[17] 7 days, 1 month, 3 months and 1 year after the stroke. Handicap level was assessed by the Oxford Handicap Scale ''Rankin''[18] at 1 month, 3 months and 1 year after the ischaemic stroke. In the case of death, the date and reason were registered.

Abbreviations: rhIL1ra, recombinant human interleukin 1 receptor antagonist; SSS, Scandinavian Stroke Scale; VNTR, variable number tandem repeat

Genetic analysis

Peripheral blood samples were collected once from each patient during the follow-up period. Genomic DNA was isolated with the use of Tri-Reagent (Sigma). Genotyping was performed by polymerase chain reaction as described previously.[19]

Statistical analysis

Allele frequencies were calculated by allele counting. Hardy–Weinberg equilibrium was tested by a C^2 goodness-of-fit test. Categorical variables were compared by the χ^2 test or Fisher's exact test. Continuous variables were compared by analysis of variance with retrospective testing using the Scheffe test (normal distribution), or by Kruskal–Wallis analysis of variance with retrospective testing using the Mann–Whitney U test (not normal distribution). Previous studies emphasised that intervals between points used in the Barthel Scale and the Rankin Scale are not necessarily equal,[20] and it would therefore be more appropriate not to analyse them as continuous variables. Consequently, the Barthel Scale, the Rankin Scale and the SSS were evaluated, dichotomising the scale, by the χ^2 test. The selected categories were 0–14 and 15–20 for the Barthel Scale, 0–3 and 4–5 for the Rankin Scale, and 0–25 and 26–58 points for the SSS. Comparisons were made using a 95% confidence interval. For multiple comparisons, hypothesis testing was performed using the Bonferroni correction (p value divided by the total number of pairwise comparisons) to correct for the possibility that in multiple comparisons, the null hypothesis would be rejected by chance. Multiple regression analyses were also performed to evaluate the role of *IL1RN* genotypes in predicting the risk of death after ischaemic stroke. Variables with a statistically significant association in the univariate analysis using the Bonferroni correction were included in the regression models, taking into account baseline and clinical variables, and controlling for age (grouped into the four categories: $\leqslant$60, 61–70, 71–80 and $\geqslant$81 years). Statistical analyses were performed using STATISTICA PL 7.0 software package (StatSoft, Cracow, Poland).

RESULTS

We detected the following *IL1RN* alleles (allele frequency in parentheses): *IL1RN**1 (72.8%), *IL1RN**2 (24.5%), *IL1RN**3 (2.4%), and *IL1RN**4 (0.2%). We found no significant deviations from the Hardy–Weinberg equilibrium for the studied alleles. We failed to detect any association between carriership of one of the three most frequent *IL1RN* genotypes (*IL1RN**1/*IL1RN**1, *IL1RN**1/*IL1RN**2, and *IL1RN**2/*IL1RN**2, representing 95% of all detected genotypes) and the age at onset, and distribution of vascular risk factors. We also found no relationship between *IL1RN* genotypes and stroke subtypes according to the TOAST criteria. We found no significant differences between carriers of different *IL1RN* genotypes when comparing severity of symptoms at the time of maximum impairment and on admission. Homozygotes for the *IL1RN**2 allele showed less severe neurological and functional impairments after 7 days, 30 days, 3 months and 1 year compared with carriers of the *IL1RN**1/*IL1RN**2 and *IL1RN**1/*IL1RN**1 *IL1RN* genotypes (table 1).

Table 1 Stroke outcomes after 7 days, 1 month, 3 months and 1 year

	***IL1RN* (VNTR)**		
	***IL1RN**1/*IL1RN**1**	***IL1RN**1/*IL1RN**2**	***IL1RN**2/*IL1RN**2**
Variable	**n = 208**	**n = 133**	**n = 29**
After 7 days			
SSS, median (IQR)	46 (28)	47 (28)	52 (15)$^{0.022*}$
Severe neurological impairment (SSS $\leqslant$ 25), %	24	25	12
BS, median (IQR)	11 (16)	12 (17)	20 (15) $^{0.048*}$
Severe disability (BS $\leqslant$ 14), %	62	55	44$^{0.041*}$
Death, %	5	13$^{0.011*}$	14$^{0.051*}$
After 1 month	**n = 208**	**n = 133**	**n = 29**
SSS, median (IQR)	51 (13)	51 (19)	55 (6)$^{0.007*,\ 0.009\dagger}$
Severe neurological impairment (SSS $\leqslant$ 25), %	12	15	5
BS, median (IQR)	16 (11)	16 (13)	20 (5)$^{0.020*,\ 0.038\dagger}$
Severe disability (BS $\leqslant$ 14), %	44	49	25
Dependence (RS 4–5), %	32	32	15
Death, %	14	18	31$^{0.024*}$
After 3 months	**n = 203**	**n = 127**	**n = 28**
BS, median (IQR)	18 (7)	20 (8)	20 (0)$^{0.002*,\ 0.016\dagger}$
Severe disability (BS $\leqslant$ 14), %	30	30	0$^{0.008*,\ 0.009\dagger}$
Dependence (RS 4–5), %	18	19	6
Death, %	24	26	34
After 1 year	**n = 194**	**n = 110**	**n = 23**
BS, median (IQR)	20 (4)	20 (4)	20 (0)$^{0.007*,\ 0.036\dagger}$
Severe disability (BS $\leqslant$ 14), %	21	25	0$^{0.004*,\ 0.026\dagger}$
Dependence (RS 4 to 5), %	16	15	0
Death, %	34	37	42

BS, Barthel Scale; IQR, interquartile range; RS, Rankin Scale; SSS, Scandinavian Stroke Scale.
Pearson χ^2 test (categorical data); Mann–Whitney U test (continuous data).
p Value for significance using the Bonferroni correction is 0.003 for analyses after 7 days and 1 month, and 0.004 for analyses after 3 months and 1 year.
*p Value for the comparison with the *1/*1 group.
†p Value for the comparison with the *1/*2 group.

Table 2 *IL1RN* alleles and the risk of death during the first 7 or 30 days after ischaemic stroke

Model	OR (95% CI)	p Value
Death during 7 days: IL-1RN*2		
Model 1: Univariate	3.07 (1.49 to 6.32)	0.002
Model 2: Adjusted for age, CHF, coronary artery disease, diabetes	3.25 (1.47 to 7.23)	0.003
Model 3: Adjusted for age, severe consciousness disturbance at maximum impairment, SSS score on admission	4.38 (1.84 to 10.40)	0.001
Model 4: Adjusted for age, CHF, coronary artery disease, diabetes, severe consciousness disturbance at maximum impairment, SSS score on admission	4.86 (1.88 to 12.56)	0.001
Death during 1 month: IL1RN*2/*2		
Model 1: Univariate	2.39 (1.03 to 5.54)	0.041
Model 2: Adjusted for age, CHF, diabetes	2.83 (1.11 to 7.21)	0.029
Model 3: Adjusted for age, severe consciousness disturbance, SSS score on admission	3.82 (1.39 to 10.50)	0.009
Model 4: Adjusted for age, CHF, diabetes, SSS score on admission	4.25 (1.46 to 12.35)	0.008

CHF, congestive heart failure; OR, odds ratio; SSS, Scandinavian Stroke Scale
Model 2 includes *IL1RN* genotype adjusted for significant predictors of death in multivariate analysis including age, sex, and vascular risk factors: hypertension, atrial fibrillation, congestive heart failure, coronary artery disease, diabetes, previous myocardial infarct, previous stroke, previous transient ischaemic attack, cigarette smoking, heavy alcohol intake.
Model 3 includes *IL1RN* genotype adjusted for significant predictors of death in multivariate analysis including age and variables indicating the initial stroke severity: severe disturbance of consciousness at maximum impairment, Scandinavian Stroke Scale score on admission, pre-stroke handicap.
Model 4 includes all significant predictors of death in models 2 and 3.

More patients with one or two copies of the *IL1RN**2 allele died in the first 7 days after a stroke than *IL1RN**1/*IL1RN**1 homozygotes (table 1). After adjustment for other significant predictors of 1-week post-stroke mortality, the estimated odds ratio for death as a function of carrying at least one *IL1RN**2 allele was 4.86 ($p = 0.001$; table 2).

Patients with two copies of the *IL1RN**2 allele had significantly higher mortality within 30 days after the stroke than those with *IL1RN**1/*IL1RN**1 or *IL1RN***1/*IL1RN**2 ($p = 0.008$; table 1). In a regression analysis, homozygotes for the *IL1RN**2 allele had increased risk of death within the first month after the ischaemic stroke (table 2).

DISCUSSION

Our data suggest that homozygotes for the *IL1RN**2 allele are likely to have less severe neurological and functional impairment after ischaemic stroke *IL1RN**1/*IL1RN**2 heterozygotes and *IL1RN**1/**IL1RN*1 homozygotes.

Neurological and functional impairments after a stroke reflect ischaemia-induced brain tissue damage, established to be markedly influenced by post-ischaemic brain inflammatory cascade.[1 2] An important part of the inflammatory process induced by brain ischaemia is a rapid and extensive synthesis of IL1.[6 7] It has been clearly established that microglial cells as well as peripheral blood monocytes have an important role in post-ischaemic inflammation, which are activated and infiltrate into ischaemic brain tissue.[1 2] The *IL1RN**2 allele is associated with increased IL1ra production in human monocytes.[9] Microglial cells are thought to be resident brain macrophages, so it is highly likely that the effect of the *IL1RN**2 allele is the same in these cells as in peripheral blood monocytes. Thus, increased IL1ra production in both microglia and peripheral blood monocytes of *IL1RN**2-homozygotic patients with stroke is possibly associated with anti-inflammatory phenotype resulting in decreased secondary brain tissue damage, and consequently in a better neurological and functional outcome. Such a result is consistent with results of animal studies.[6 7]

In our study, the *IL1RN**2 allele carriers had an increased risk of death within the first week after the ischaemic stroke, and homozygotes for this allele had increased risk of death within 30 days. These two issues should be considered when interpreting such an observation. Firstly, IL1 has a major role in determining the effectiveness of the immune response to infections, which are common (21–65% of patients[21]) fatal complications after an ischaemic stroke. Possibly, increased production of IL1ra in blood monocytes of *IL1RN**2-positive patients may explain their increased susceptibility to infections. In a clinical trial,[8] treatment of patients with stroke with intravenous rhIL1ra was associated on the one hand with better neurological and functional outcome, and on the other, with increased incidence of serious infections. Secondly, in endothelial cells, the homozygotic genotype *IL1RN**2/*IL1RN**2 was associated with 2-3-fold less IL1ra protein production than the common genotype *IL1RN**1/*IL1RN**1.[10] Thus, the *IL1RN**2 allele may favour accelerated intravascular inflammatory processes, followed by enhanced intravascular coagulation[22] and increased production of potent vasoconstrictors such as endothelin.[23] The net effect of these events may be an increased risk of vascular complications in post-stroke *IL1RN**2-positive patients. Compared with patients homozygotic for *IL1RN**1, those with the *IL1RN**2 allele were more likely to die from infectious complications (*IL1RN**2/*IL1RN**2, 18%; *IL1RN**1/*IL1RN**2, 16%; *IL1RN**1/*IL1RN**1, 12%) and from cardiovascular complications (27%, 23% and 19%, respectively). Patients homozygotic for *IL1RN**2 were most likely to die from pulmonary embolus (18%, 5% and 6%, respectively), and less likely to die from neurological sequelae of the first stroke (18%, 32% and 34%, respectively). However, owing to the low number of patients who died from individual reasons, these results do not allow us to definitely conclude what mechanisms underlie an association of *IL1RN**2 with increased risk of death within 1 month of ischaemic stroke.

However, the possibility of a type I statistical error could not be excluded in our data analyses and therefore the over-interpretation of the study results as causal relationships, these may be only accidental associations. A limitation of our study is the small sample size, especially the low number of patients homozygotic for the *IL1RN**2 allele. Despite the small sample size, the observation of an odds ratio of about 4 for the *IL1RN* genotype and risk of death within 7 days and 1 month after stroke suggests an association of notably strong magnitude, which would be of interest to confirm in future studies.

In future, anti-inflammatory treatments, especially those aimed at modulating inflammatory cytokine-mediated inflammation, are supposed to be useful in treating ischaemic stroke. So, the discovery of *IL1RN* VNTR and other cytokine gene polymorphisms associated with severity and outcome in

ischaemic stroke might someday be applied in pharmacogenomic strategies. For example, our results allow us to hypothesise that the effects of treatment with rhIL1ra in acute stroke may be different in patients with different genotypes of *IL1RN* VNTR. So, genotyping of this polymorphism may be useful in further planning or in interpreting results of clinical trials with rhIL1ra in the treatment of stroke.

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