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Functional Polymorphisms in Toll-like Receptor 4 Are Associated With Worse Outcome in Acute Ischemic Stroke Patients

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Abstract

Toll-like receptor-4 (TLR4) is important in neuroinflammation. Single nucleotide polymorphisms (SNPs) in *TLR4*, including 1063 A/G [Asp299Gly] and 1363 C/T [Thr399Ile], are associated with altered immune responses but their effect on acute ischemic stroke (AIS) outcome is unknown. We collected demographic, clinical, laboratory, radiologic and genotype data on 113 AIS patients and performed multivariate analyses to assess associations between *TLR4* SNP haplotype and either neurological outcome, infection or inflammatory markers. In adjusted analyses, *TLR4* SNPs were associated with worse outcome as well as increases in circulating leukocytes, C-reactive protein and interleukin-1 receptor antagonist. In AIS, variations in *TLR4* may influence neurological outcome.

Keywords

Toll-like receptor 4 (TLR4); single nucleotide polymorphisms (SNP); stroke; outcome; infection

Acute ischemic stroke (AIS) induces profound alterations in both systemic and central nervous system (CNS) immune responses [1]. In the periphery, these changes include robust increases in plasma concentrations of pro-inflammatory markers such as C-reactive protein (CRP) and interleukin (IL)-6 [1]. In brain, this response includes production of pro-inflammatory cytokines such as IL-1 β , IL-6 and tumor necrosis factor- α (TNF- α) from astrocytes and microglia [2] as well as a marked influx of leukocytes into the ischemic hemisphere [2].

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(For video abstract, please see supplemental digital content file)

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Toll-like receptors (TLRs) are a family of pattern recognition receptors involved in identification of, and response to, foreign pathogens [3]. In brain, TLRs are expressed by microglia and astrocytes [2] and are critical in initiation of innate immune response to injury [3]. Thirteen TLRs have been identified and each recognizes different pathogen-associated molecular patterns (PAMPs) including bacterial cell wall/membrane components such as lipotechoic acid (TLR2) and lipopolysaccharide (LPS or endotoxin) (TLR4) [3]. Activation of TLRs by endogenous ligands (also known as danger associated molecular patterns or DAMPs) released from ischemia-injured cerebral vasculature and parenchyma is a possible mechanism for initiation of both inflammatory and immunomodulatory responses in AIS [2, 4]. A number of TLR4-activating DAMPs have been identified in brain including heat shock proteins (HSPs), high-mobility group box 1 (HMGB1) and peroxiredoxins [2, 4]. In animal models, TLR4 signaling has been implicated in post-stroke neuroinflammation and injury [5, 6]. TLR4 is also critical in the robustly neuroprotective ischemic preconditioning phenomenon [7].

Single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in the immune response can influence clinical outcomes following AIS [8]. There are approximately twenty known SNPs in *TLR4* [9]. Two of these *TLR4* SNPs, 1063 A/G (Asp299Gly) and 1363 C/T (Thr399Ile), occur at significant frequencies (>5%) in populations across the globe [10]. In Caucasians, these two *TLR4* SNPs co-segregate; thus the double SNP 299/399 haplotype occurs more frequently than either of the individual SNP haplotypes (299/*wt* or *wt*/399) [9, 10]. Functionally, SNPs at either or both loci result in TLR4 proteins with altered ligand binding domains [11] and the 299/399 haplotype is associated with hypo-responsiveness to LPS in some [9, 12], but not all [13], prior studies. In addition, the 299/399 haplotype is associated with increased risk of systemic infection [10, 14]. The effect of these *TLR4* SNPs on post-stroke infection and clinical outcome is unknown.

METHODS

Research Subjects

The patient population for this study was described elsewhere [15, 16]. The University of Washington's (UW) Institutional Review Board approved this study. Patients or their surrogates provided informed consent.

Data collection

Demographic, clinical and radiological data were collected on all patients. Stroke severity at time of presentation was quantified using the National Institutes of Health Stroke Scale (NIHSS) score. Total infarct volume on initial diffusion weighted magnetic resonance imaging (MRI) was calculated by the ABC/2 method [17]. Stroke sub-types were classified as described [18]. Stroke outcome was determined at 3 months by the modified Rankin Scale (mRS); poor outcome was defined as mRS>2. Infections included in our analyses were in-hospital urinary tract infections (UTI) and pneumonia (PNA). Clinical criteria for these infections were as previously defined [15]. All patients with infection were treated with antibiotics.

Laboratory studies

Leukocyte counts and plasma CRP levels were determined by the hospital laboratory. Concentrations of circulating cytokines [IL-6, IL-10, TNF α , and IL-1 receptor antagonist (IL-1ra)] were measured with a cytometric bead-based system (Fluorokine® MAP R&D Systems). Values below the specific limit of detection for each cytokine were assigned a value equivalent to the lower level of detection.

TLR4 SNP genotyping

Prior studies examining the 1063 A/G (Asp299Gly) and 1363 C/T (Thr399Ile) *TLR4* loci have used different groupings of the variant haplotypes (299/*wt*, *wt*/399 and 299/399) to demonstrate significant associations with clinical parameters. Some have looked at the 299/399 haplotype alone [13, 14] whereas others have looked at associations with any of the three variant haplotypes combined (299/*wt*, *wt*/399 or 299/399) [9, 10]. The two approaches have complementary scientific merit [9] and we chose to do both throughout this study although the data presented in the tables show only results with the combined haplotypes (299/*wt*, *wt*/399 or 299/399). DNA extraction from blood plasma samples, qRT-PCR and allelic discrimination analyses were performed as described [19]. Target SNP reference identification numbers were rs4986790 and rs4986791. All samples were processed in triplicate. Reproducibility of the genotyping method was confirmed as described [19].

Statistical analyses

Descriptive data are presented as median and interquartile range (IQR) for continuous variables and percentages for categorical variables. Group comparisons were performed using the Mann-Whitney *U* test or the χ^2 test statistic as appropriate. Logistic regression was used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the effect of the *TLR4* SNPs on infection by day 15 and on neurologic outcome at 3 months. With a relatively small sample as in this study, there is only good statistical power for detecting a large effect. For example, for the association of genotype with clinical outcome (Table 2A) the power is 32%, 59%, and 73% for detecting an OR of 5, 10, or 15, respectively. For the association of genotype with infection (Table 2B) the power is 51%, 79%, and 90% for detecting an OR of 5, 10, or 15, respectively. For table 3, there is 85% power for detecting a difference of 1.0 Standard Deviation (SD) between groups.

RESULTS

Of the 113 patients in the study, 10 (8.8%) were heterozygous for a haplotype that included either one or both of the indicated *TLR4* SNPs: 8 (7.1%) had the double SNP (299/399 haplotype) and 2 (1.8%) had the single SNP (*wt*/399 haplotype). The remaining 103 (91.1%) patients had neither SNP (*wt*/*wt* haplotype). The observed genotype frequencies for both SNPs were in Hardy-Weinberg equilibrium (<http://www.oege.org/>).

There were no significant differences in baseline characteristics or medical history of patients based on whether or not they had a defined *TLR4* SNP (i.e. *wt*/*wt* vs. 299/*wt*, *wt*/399 or 299/399) (Table 1). Neither initial stroke severity nor infarct volume differed between patients with a *TLR4* SNP and those without (Table 1). Patients with a *TLR4* SNP haplotype,

however, were more likely to have lacunar stroke and less likely to have cardioembolic stroke than patients with the *wt/wt* haplotype (Table 1). Rates of infection were similar among all patients irrespective of *TLR4* haplotype (Table 1). Table 1 shows only results for patients who had any of the three *TLR4* SNP-positive haplotypes investigated, although results were not significantly different when only patients with the co-segregating dual SNP 299/399 alone, were included in the analysis.

Table 2A displays the association between *TLR4* SNP haplotype and poor neurological outcome (mRS>2) at 3 months. Results are shown both unadjusted and adjusted for variables known to affect stroke outcome. Table 2A shows that there was no association between the presence of a *TLR4* SNP and poor outcome in the unadjusted analysis. However, after adjusting for initial stroke severity and age, the presence of a *TLR4* SNP was associated with poor outcome. Neither further adjustment for stroke etiology nor the presence of infection significantly altered this association. Results for the 299/399 SNP haplotype alone were largely similar to those for the three variant *TLR4* SNP haplotypes combined: unadjusted, OR $\pm$ 95%CI = 1.47 [0.35-6.27], *p* = NS; adjusted for initial stroke severity and age, 10.79 [1.32-88.4], *p* = 0.027; adjusted further for stroke etiology and infection, 12.61 [1.42-111.9], *p* = 0.023.

Overall, 29/113 (25.7%) of patients in this cohort had an infection by day 15 after AIS (Table 1). Having one or both *TLR4* SNPs was not associated with an increased likelihood of infection in the unadjusted analysis, (OR $\pm$ 95%CI = 1.25 [0.30-5.23], *p* = NS) (Table 2B). After controlling for initial stroke severity and age there was still no significant association found between the presence of a *TLR4* SNP-containing haplotype and the risk of infection, 2.22 [0.44-11.20], *p* = NS (Table 2B). Similarly, no significant association was found between the 299/399 haplotype alone and risk of infection, 2.06 [0.30 – 14.33], *p* = NS, post adjustment for stroke severity and age.

Patients with a haplotype containing either one or both *TLR4* SNPs had higher white blood cell (WBC) counts at 3 days and 1 week post AIS (Table 3); this elevation was independent of stroke severity. A similar pattern of WBC elevation was seen for the 299/399 haplotype alone (**data not shown**). The increase in WBC counts was driven by an increase in polymorphonuclear (PMN) cell counts (Table 3). No significant changes in either lymphocyte or monocyte counts were found with any of the variant *TLR4* haplotypes examined. Plasma CRP was also elevated in the SNP-positive patient group at 3 months after stroke onset. This increase in CRP at 3 months was also seen in the 299/399 haplotype alone (**data not shown**). Of the cytokines assayed (IL-6, IL-10, TNF α and IL-1ra), the only significant difference between SNP-positive and *wt/wt* patients was for IL-1ra, which was higher in the SNP-positive patient group at the one-week time point (Table 3).

DISCUSSION

This study demonstrates that two functionally significant *TLR4* SNPs were associated with poor neurological outcome following AIS. At three months after stroke, the group of patients with either one or both of the *TLR4* SNPs (299/*wt*, *wt*/399 or 299/399 haplotypes) had significantly worse clinical outcomes. This association persisted when limiting the

analysis to patients with the 299/399 haplotype alone and also in multivariate models controlling for age, stroke severity, stroke etiology and infection. The *TLR4* SNP patient group also exhibited transiently increased WBC counts and plasma levels of CRP and IL-1ra.

The two *TLR4* SNPs investigated are in strong linkage disequilibrium in Caucasians [9, 14]. Our results are consistent with this in that eight of the ten *TLR4* SNP-positive patients were heterozygous for the 299/399 dual SNP haplotype. The 299/399 haplotype is associated with increased rates of systemic infection [10, 14], however we did not find an association with infection in our cohort. Because the rates of post-stroke infection are generally high, the association between these SNPs and infection risk may have been overwhelmed by stroke related factors. Given the limited statistical power of this study, it is also possible that the lack of a demonstrated association between genotype and infection could be due to a type II (false negative) error.

Despite the absence of a demonstrated association between *TLR4* SNP haplotype and infection in our cohort, the possibility that poor outcomes in *TLR4* SNP patients could be related to infection was considered. Post-stroke infection is an independent risk factor for poor outcome [1] and worse outcome was seen among patients with post-stroke infection in our cohort [16]. Our data, however, showed that adjustment for infection had little impact on the association between *TLR4* SNP haplotype and clinical outcome (Table 2A). Thus post-stroke infection is unlikely to explain this association. It is important to note that our modest sample size and multiple comparisons mandate caution in the interpretation of these results. However, the primary association between *TLR4* SNP haplotype and clinical outcome here was a singular pre-specified *a priori* hypothesis. Thus the possibility of a type 1 (false positive) error is reduced [20]. Nevertheless, our findings will need to be replicated in an independent cohort of AIS patients.

Given the findings in Table 2A, a mechanism to account for the effect of *TLR4* SNP haplotype on outcome, independent of infection, should be proposed. Our findings seem to contrast with those in rodent studies where spontaneous mutations in [6] or complete absence of [21] the *TLR4* gene results in reduced infarct volumes and improved neurobehavioral outcomes. The *TLR4* SNPs investigated here, however, result in structural alterations in human TLR4 that are different than in mice [12]. The responses of these SNP-altered TLR4 proteins to endogenous ligands (or DAMPs) released from ischemic brain tissue are unknown. The extent of injury and recovery in the brain following stroke may be modulated by the TLR4-response of resident microglia and/or infiltrating macrophages to DAMPs [2, 4]. Alternatively, modification of TLR4 function in the immune cells of SNP-positive patients could result in an attenuation of baseline TLR4-dependent “preconditioning” [7].

Experimental data support a beneficial role for the anti-inflammatory cytokine IL-1ra in stroke [22]. In a recent study characterizing the same patient cohort as described here, elevated plasma IL-1ra was independently associated with an increased risk of post-stroke infection but not clinical outcome [16]. Our data indicate that patients with a *TLR4* SNP haplotype have transiently increased IL-1ra plasma levels. This association persisted after

controlling for stroke severity (Table 3) and was not significantly altered by adjustment for infection (data not shown). The biological explanation for this association, independent of infection, is uncertain. However the *TLR4* SNPs studied here alter both plasma levels of cytokines [23] and immune cell release of cytokines following stimulation with TLR4 agonists [10, 12]. Thus, the IL-1ra finding here is consistent with previous literature.

Increased WBC counts and elevated plasma CRP are independently associated with poor outcome after stroke [1]. Our finding that variant *TLR4* SNPs are associated with transient increases in both WBC count and CRP is interesting and could help explain the *TLR4* SNP effect on outcome. As with IL-1ra, the associations with both WBC count and CRP persisted after adjusting for infection (data not shown). The biological explanations for these associations are also uncertain. However, our findings here are again consistent with prior work [24, 25].

CONCLUSION

Functionally significant variations in *TLR4* are associated with: (i) worse neurological outcome and (ii) alterations in systemic markers of inflammation following AIS. These data are the first to suggest a direct connection between TLR4 function and stroke pathophysiology in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

	TLR4 Haplotype		
Parameter	wt/wt N=103	299/wt, wt/399 or 299/399 SNP N=10	P
Baseline Characteristics			
age	57 (46-67)	46 (42-64)	0.31
gender (female)	33 (32%)	5 (50%)	0.25
Medical History			
AF	16 (16%)	0 (0%)	0.18
CHD	26 (25%)	1 (10%)	0.28
DM	23 (22%)	4 (40%)	0.21
HTN	53 (51%)	7 (70%)	0.26
smoker	40 (39%)	2 (20%)	0.24
HLD	74 (72%)	7 (70%)	0.90
Clinical/Radiological Data on Patients' Stroke			
initial NIHSS score	11 (4-20)	9 (3-18)	0.46
initial infarct volume (cc)	12 (2-90)	12 (0.1-117)	0.46
Stroke Etiology			
lacunar	8 (8%)	3 (30%)	0.02
cardioembolic	30 (29%)	0 (0%)	0.046
large artery atherosclerosis	16 (16%)	1 (10%)	0.64
dissection	6 (6%)	1 (10%)	0.60
Other[#]	21 (20%)	2 (20%)	0.76
unknown	22 (21%)	3 (30%)	0.53
Infections			
all infections	26 (25%)	3 (30%)	0.76
PNA	12 (12%)	0 (0%)	0.33

AF=atrial fibrillation, CHD=coronary heart disease, DM=diabetes mellitus, HTN=hypertension, HLD=hyperlipidemia, NIHSS=National Institutes of Health Stroke Scale, PNA=pneumonia, wt=wild type, SNP=single nucleotide polymorphism.

NS = p>0.20,

[#] Other etiology category included 1 iatrogenic, 3 paradoxical emboli and 19 with either multiple competing etiologies or uncertain etiology

Table 2A

Association between TLR4 SNP (1063 A/G [Asp299Gly] and/or 1363 C/T [Thr399Ile]) and poor outcome (modified Rankin Scale score >2) at 3 months post AIS.

Presence of TLR4 SNP predicts poor outcome		
model adjusted for:	OR (95% CI)	P
TLR4 SNP *	2.21 (0.58-8.38)	0.24
TLR4 SNP+NIHSS	9.22 (1.43-59.5)	0.02
TLR4 SNP+NIHSS + age	12.92 (1.78-93.90)	0.01
TLR4 SNP + NIHSS + age + stroke etiology	15.46 (2.04-117.04)	0.008
TLR4 SNP + NIHSS + age + stroke etiology + infection	14.16 (1.75-114.81)	0.01

* Numerical breakdown of unadjusted data:

99/113 (88%) of patients in study had outcome data available at this time point.

42/99 (42%) of patients with available outcome data at this time point had poor outcome.

6/10 (60%) of patients with available outcome data at this time point **and** a TLR4 SNP had a poor outcome

36/89 (40%) of patients with available outcome data at this time point **without** a TLR4 SNP had a poor outcome

Table 2B

Association between TLR4 SNP (1063 A/G [Asp299Gly] and/or 1363 C/T [Thr399Ile]) and infection at 15 days post AIS.

Presence of TLR4 SNP predicts infection		
model adjusted for:	OR (95% CI)	<i>P</i>
TLR4 SNP	1.25 (0.30-5.20)	0.76
TLR4 SNP + NIHSS	2.08 (0.41-10.52)	0.38
TLR4 SNP + NIHSS + age	2.22 (0.44-11.20)	0.34

Abbreviations: = AIS = Acute ischemic stroke, NIHSS = National Institutes of Health Stroke Scale, SNP= single nucleotide polymorphism

Table 3

Markers of inflammation from AIS onset to 3 months in patients who either had or did not have one of the variant TLR4 SNP haplotypes under investigation here (299/*wt*, *wt*/399 or 299/399): indicated in table as “+SNP”. Data values shown are medians (interquartile range). *P* values are adjusted for stroke severity.

Time:	72 hours			1 week			1 month			3 months		
	<i>wt</i> / <i>wt</i>	+SNP	<i>P</i>	<i>wt</i> / <i>wt</i>	+SNP	<i>P</i>	<i>wt</i> / <i>wt</i>	+SNP	<i>P</i>	<i>wt</i> / <i>wt</i>	+SNP	<i>P</i>
WBCs (thou/microL)	7.44 (5.99, 10.00)	8.97 (7.68, 12.42)	0.025	7.66 (6.02, 9.78)	10.70 (7.46, 13.00)	0.003	6.53 (5.34, 8.54)	8.43 (6.82, 11.15)	0.074	6.88 (5.42, 8.12)	7.99 (7.41, 10.10)	0.139
PMNs (thou/microL)	5.04 (3.44, 6.87)	6.08 (5.06, 8.06)	0.023	4.80 (3.36-6.44)	5.49 (3.95, 8.26)	0.104	4.12 (3.00, 5.43)	5.18 (4.22-7.33)	0.087	3.89 (3.00, 5.43)	5.48 (4.10, 7.30)	0.059
lymphs (thou/microL)	1.47 (1.22, 1.82)	1.71 (1.42, 1.94)	0.755	1.80 (1.40-2.25)	1.80 (1.69, 2.13)	0.918	1.78 (1.27-2.04)	1.96 (1.63, 2.45)	0.175	1.73 (1.34, 2.14)	1.83 (1.41, 2.59)	0.468
monos (thou/microL)	0.64 (0.46, 0.86)	0.61 (0.46, 0.82)	0.742	0.66 (0.50, 0.89)	0.68 (0.58, 1.04)	0.363	0.52 (0.42, 0.68)	0.50 (0.47, 0.60)	0.727	0.50 (0.41, 0.65)	0.59 (0.52, 0.67)	0.127
CRP (mg/L)	9.5 (2.6, 48.1)	8.8 (1.9, 87.1)	0.208	7.20 (2.20, 25.95)	8.20 (3.85, 48.65)	0.811	2.70 (0.95, 9.15)	4.00 (0.95-10.92)	0.900	1.90 (0.60, 5.90)	2.50 (0.90, 12.00)	0.002
IL-6 (pg/mL)	1.94 (nd, 7.23)	1.66 (nd, 15.16)	0.941	1.26 (nd, 4.39)	2.87 (1.47, 4.83)	0.999	nd (nd, 2.12)	nd (nd, 2.52)	0.817	nd (nd, 1.62)	1.41 (nd, 1.69)	0.611
IL-10 (pg/mL)	nd (nd, 0.93)	nd (nd, nd)	0.182	nd (nd, 0.75)	nd (nd, 0.56)	0.519	nd (nd, 0.70)	nd (nd, nd)	0.301	nd (nd, 0.70)	nd (nd, nd)	0.369
TNFα (pg/mL)	1.87 (nd, 2.96)	nd (nd, 4.05)	0.669	2.12 (nd, 3.92)	nd (nd, 4.72)	0.069	1.88 (nd, 3.89)	2.18 (nd, 4.30)	0.519	1.98 (nd, 3.26)	1.76 (nd, 4.77)	0.827
IL-1ra (pg/mL)	1645 (98, 2946)	2401 (765, 4137)	0.094	1777 (981, 3189)	2086 (1458, 8401)	0.009	1400 (812-3214)	2029 (1448, 3150)	0.272	1009 (579, 1745)	1238 (606, 2579)	0.982

SNP=single nucleotide polymorphism, AIS=acute ischemic stroke, WBCs=white blood cells, PMNs=polymorphonuclear cells, monos = monocytes, lymphs=lymphocytes, CRP=high sensitivity C reactive protein, IL=interleukin, TNF=tumor necrosis factor, IL-1ra=IL-1 receptor antagonist, *wt*=wild type, nd=not detected, NS=P>0.20, P=p-value (adjusted for initial stroke severity) for comparisons between *wt*/*wt* haplotype vs. either the 299/*wt*, *wt*/399 or 299/399 haplotypes.