Genetic Variants and Susceptibility to Neurological Complications Following West Nile Virus Infection

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To determine genetic factors predisposing to neurological complications following West Nile virus infection, we analyzed a cohort of 560 neuroinvasive case patients and 950 control patients for 13,371 mostly nonsynonymous single-nucleotide polymorphisms (SNPs). The top 3 SNPs on the basis of statistical significance were also in genes of biological plausibility: rs2066786 in RFC1 (replication factor C1) ($P = 1.88 \times 10^{-5}$; odds ratio [OR], 0.68 [95% confidence interval {CI}, .56–.81]); rs2298771 in SCN1A (sodium channel, neuronal type 1 α subunit) ($P = 5.87 \times 10^{-5}$; OR, 1.47 [95% CI, 1.21–1.77]); and rs25651 in ANPEP (ananyl aminopeptidase) ($P = 1.44 \times 10^{-4}$; OR, 0.69 [95% CI, .56–.83]). Additional genotyping of these SNPs in a separate sample of 264 case patients and 296 control patients resulted in a lack of significance in the replication cohort; joint significance was as follows: rs2066786, $P = .0022$; rs2298771, $P = .005$; rs25651, $P = .042$. Using mostly nonsynonymous variants, we therefore did not identify genetic variants associated with neuroinvasive disease.

Over the past decade, West Nile virus (WNV) has emerged as an important human pathogen in North America, where it has been reported in a majority of states and provinces in the United States and Canada [1–3]. Of those infected, approximately 20% develop mild symptoms (“West Nile fever,” eg, fever, malaise, headache, myalgia, rash) [4] and about 1 in 150 develop meningitis or encephalitis [4, 5]. The incidence of severe neurological syndromes increases with age and includes encephalitis, meningitis, acute flaccid paralysis, peripheral neuropathy, polyradiculopathy, optic neuritis, and acute demyelinating encephalitis [5–9]. Pathological changes within the central nervous system appear to be due to several factors, including the direct result of viral proliferation within neuronal and glial cells, cytotoxic immune response to infected cells, diffuse perivascular inflammation, and microglial nodule formation [10–12].

Given the fact that only a small minority of those infected develop severe disease and the fact that risk factors, apart from older age and immunosuppression [1, 13–15], are not well defined, there is a strong rationale to suspect a genetic predisposition to WNV neurological complications. Several putative susceptibility genes have been reported in single candidate gene studies. An association between symptomatic WNV disease and homozygosity for the CCR5A32 mutation in the chemokine receptor gene CCR5 was initially reported [16, 17]. More recently, this association was not replicated, but a study suggestive of a link to clinical manifestations of infection with CCR5Δ32 mutation was reported [18]. The plausibility of a susceptibility
locus in the oligoadenylate synthetase (OAS) gene cluster was reported [19, 20]. More recently, a candidate gene study examining the OAS gene cluster alone suggested a predisposition to WNV infection [21].

Because exposure to WNV is difficult to control for, we sought to examine genetic susceptibility to complications given documented WNV infection. We hypothesized that the use of coding nonsynonymous markers would result in the discovery of ≥1 genetic variants associated with severe WNV disease that may be directly likely to lead to a causal mechanism. We report an association between the replication factor C1 gene, RFC1, and severe WNV disease in the first phase of association testing. We found no association between CCR5A32 mutation and a susceptibility to severe complications to WNV infection. Similarly, we found no association between the OAS gene cluster and severe WNV disease. Our results indicate that RFC1 may potentially play a role in the etiology of severe WNV illness.

PARTICIPANTS AND METHODS

Study Participants
During the period September 2004 through December 2009, we enrolled participants from states and provinces in the United States (Nebraska, Pennsylvania, Texas, and Colorado) and Canada (Alberta and Saskatchewan) that were among those with the highest number of individuals with severe WNV disease. Individuals were eligible if they were aged ≥18 years and had evidence of WNV infection on the basis of Centers for Disease Control and Prevention diagnostic laboratory criteria [22]. Case patients were defined as patients who met criteria for WNV infection along with modified case definitions for meningitis, encephalitis, or acute flaccid paralysis [9]. Control participants were individuals who met criteria for infection but who did not meet clinical case definitions for meningitis or encephalitis. Because the majority of testing for WNV is performed centrally by state or provincial laboratories, the state or provincial health departments contacted persons with laboratory-confirmed WNV infection by letter and by telephone to see if they were willing to be approached about the study. Clinical information about WNV infection was obtained by contacting patients’ physicians and through review of medical records. The study received institutional review board approval from McMaster University, McGill University, Nebraska Medical Center, University of Pennsylvania, and University of Texas at San Antonio.

Genotyping
Blood samples were collected at the time of enrollment and genomic DNA obtained with the use of a DNA isolation kit (Qiagen). For the first stage of the study, genotyping was conducted using the Illumina HumanNS-12 BeadChip, based largely on single-nucleotide polymorphisms (SNPs) selected by the Wellcome Trust Case Control Consortium that included all known nsSNPs with >1% minor allele frequency in European populations at the time of study design [23]. The Illumina HumanNS-12 chip included 13 371 SNPs, of which the majority were nonsynonymous variants but also included synonymous, untranslated, and tagSNPs. For the validation and replication stage, 2 panels of 34 SNPs each were designed using Sequenom MassARRAY IPLEX Gold; these included 47 SNPs with the highest association in the derivation cohort at 1 of 2 time points; 15 tagSNPs to fine map the gene that showed the highest association in the derivation cohort; 5 coding nonsynonymous SNPs uncovered in genes that were sequenced; and CCR5A32 (rs333), a 32–base pair (bp) deletion in CCR5 that was shown to be associated with clinical symptoms of WNV infection [18]. Duplicate samples were included, and laboratory technicians were blind to the case status of samples.

Sample Quality Control
Illumina HumanNS-12 Array. There were 1677 unique samples that were genotyped. Twenty samples were present in duplicate, and 1 was in triplicate. Four Centre d’Etude du Polymorphisme Humain controls were genotyped multiple times. Three duplicate samples that did not correspond to the same DNA were removed from further analysis. From the remaining samples, we calculated a pairwise reproducibility rate of 99.9926% before applying any filters on the markers. We computed the mean number of alleles shared identical by state in all possible pairs of individuals to detect population structure and cryptic relatedness in the sample. The set of SNPs used to compute this distribution was restricted to the autosomes and was selected in such a way that no 2 SNPs were allowed to be in linkage disequilibrium with each at r^2 > 0.2, in any window of 50 consecutive SNPs. To minimize admixture and other population effects, we restricted the genetic studies to samples of Caucasian origin. We excluded samples obtained from individuals who were not of self-declared non-Hispanic white ancestry, and we conducted a multidimensional scaling analysis comparing samples from the present study to HapMap samples derived from 3 populations [24] in order to identify samples from individuals whose ancestry was likely of non-Caucasian origin. Pairs of individuals who were identified as being likely related were broken up; we kept the case patient if both members of the pair were phenotypically discordant; otherwise, we kept the first participant enrolled. Seemingly related pairs involving 2 participants from distinct collection centers were discarded, on the basis that this was caused by either a sample switch or a contamination. Using this strategy, 29 individuals (17 control patients, 3 case-control pairs due to switches, and 3 case-control pairs with shared DNA) were excluded. Using all the SNPs on the X chromosome, we found inconsistencies regarding the sex of 6 individuals, who were excluded from the analysis. Samples that did not achieve a 90% call rate were excluded from the analysis.
Sequenom Assays. In a second stage, 2 panels of 34 SNPs were each genotyped in a total of 2350 unique DNA samples, a number that includes the 1677 samples genotyped on the Illumina HumanNS-12 array for the primary analysis. The samples that were removed in the primary analysis (except those that were removed for reason of low call rate) were also removed in the second stage. Samples that were not obtained from participants of self-declared non-Hispanic white ancestry were excluded. Samples that did not reach an 85% call rate in 1 panel were excluded from the analysis of that panel.

SNP Quality Control
To be included in the analysis, an SNP had to have a call rate ≥95%. Genotypic frequencies had to be consistent with the rules of Hardy-Weinberg equilibrium among control patients, at a significance level above a threshold that was chosen so that the distribution of Hardy-Weinberg significance levels among the remaining SNPs did not deviate from what was expected by chance alone (P > .0005 for the Illumina HumanNS-12 assays; P > .001 for the Sequenom assays).

Association Analysis
We used logistic regression to test for association between single SNPs and case-control status, assuming a log-additive effect of the alleles on the risk. For SNPs on the X chromosome, males were treated as homozygous females [25]. We adjusted for the collection center (ie, used as covariate) to account for possible local variations in allele frequencies or local selection. SNPs were deemed to be significant if their P values were below the 5% level, subject to a Bonferroni correction for the number of SNPs tested (P < 4.7 × 10−6, accounting for 10625 tests). Power to detect an association at this significance level was calculated using CaTS [26].

Forty-eight samples were randomly selected among the cases for resequencing with a view to fine-mapping the RFC1, SCN1A, and ANPEP exons and promoter regions. Primers were designed for amplification of the coding regions, as well as 1 kb regions upstream and downstream of the genes.

RESULTS

Nonsynonymous SNP Testing of WNV Severe Disease
We initially genotyped the Illumina HumanNS-12 array in 1677 participants: 608 case patients with neuroinvasive disease (112 meningitis, 72 encephalitis, 195 meningo-encephalitis, and 229 acute flaccid paralysis), 994 control patients, and 75 of equivocal or unknown status. There were 63 samples excluded (44 case patients, 16 control patients, and 3 of equivocal or unknown status) because of non-European ancestry (Supplementary figure 1), 26 samples excluded (2 case patients, 21 control patients, and 1 of unknown status) because of cryptic relatedness, 2 samples (1 case patient and 1 of unknown status) removed because of low call rates (<95%), 6 samples (1 case patient and 5 control patients) removed for inconsistent sex information, and 3 samples genotyped in duplicate (3 control patients) but that corresponded to different DNAs. There were 307 SNPs excluded from analysis for not reaching a sufficient call rate; 52 SNPs failed Hardy-Weinberg equilibrium at a level P < .0005; and 2387 SNPs with minor allele frequency less than 1% (including 1246 monomorphic SNPs) were excluded. The first-stage analysis was performed on 10625 SNPs in 560 case patients and 950 control patients. A summary of their characteristics is shown in Table 1.

The results from this analysis are summarized in Table 2 for all SNPs selected to undergo replication (see below). The 3 results with the highest levels of significance were from genes potentially biologically relevant: rs2066786 (P = 1.88 × 10−5; odds ratio [OR], 0.68 for the major allele [95% confidence interval [CI], .57–.81]), an exonic synonymous SNP (Pro847Pro) found in the replication factor C1 gene (RFC1 [MIM 102579]); rs2298771 (P = 5.87 × 10−5; OR, 1.47 [95% CI, 1.22–1.77]), a missense SNP (Ala1056Thr) in the sodium channel, voltage-gated, type I, α subunit gene (SCN1A [MIM 182389]); and rs25651 (P = 1.44 × 10−4; OR, 0.69 [95% CI, .56–.83]), a missense SNP (Ser752Asn) found in the alanyl (membrane) aminopeptidase gene (ANPEP [MIM 151530]). As described in detail below, each of these genes has biological plausibility, with RFC1 potentially having a role in viral replication [27], SCN1A having a role in seizure disorders [28], and ANPEP a receptor for human coronavirus [29].

Novel Coding Nonsynonymous Variants in RFC1, SCN1A, and ANPEP Regions
The 3 genes were sequenced (exons and promoter regions only) in a set of 48 randomly chosen cases (totaling 96 chromosomes), in order to find potential novel candidate risk factors. A total of 5 coding nonsynonymous variants not already genotyped were uncovered in the 3 genes (1 in RFC1, 2 in SCN1A, and 2 in ANPEP). All these novel variants showed at most 2 instances of the nonreference allele out of the 96 chromosomes, making them rare variants, each with estimated frequency <2% in the case patients. In addition, we confirmed a number of known and rare coding nonsynonymous SNPs that were already indexed in the dbSNP database at the time of the sequencing experiment.

Validation of Significant Markers
To validate findings from the first stage of the analysis, we genotyped 68 SNPs, including 47 SNPs taken on the basis of the results of the analysis of the Illumina HumanNS-12 array, at 2 different time points during the course of the project (the top 15 SNPs on the basis of a preliminary analysis of the first 445 case patients and 813 control patients collected, and the top 32 SNPs not already selected on the basis of the analysis of the complete sample, after pruning SNPs found to be in high linkage disequilibrium [r² > 0.8] with better ranked SNPs); 15 tagSNPs to try to refine the association seen in RFC1 with rs2066786,
a variant that does not change the amino acid in the amino acid sequence of the gene; and the 5 coding nonsynonymous SNPs uncovered after sequencing the RFC1, SCN1A, and ANPEP genes. In addition, we included in a panel rs333, a 32-bp deletion in CCR5 shown to be associated with increased risk of WNV infection, for which a design on the Illumina HumanNS-12 array was not available. Two control patients had insufficient call rates on both Sequenom panels and were excluded from the analysis; an additional 25 samples (6 case patients, 16 control patients, and 1 equivocal) failed only 1 Sequenom panel and were excluded only for SNPs on that panel. After excluding samples with low call rates, the SNPs were tested in the original set of (up to, depending on panel failure) 560 case patients and 950 control patients, in an additional set of 560 samples (264 case patients and 296 control patients), and in all samples jointly considered. The results are summarized in Supplementary table 1. For rs2066786 in RFC1, \( P = .58 \) in the replication set and a joint \( P = .0022 \). For rs2298771 in SCN1A, \( P = .58 \) in the replication cohort, with joint \( P = .0050 \). For rs25651 in ANPEP, the replication \( P \) value is .037, but the risk allele (the one that is more frequent among the case patients) is different from the risk allele in the original sample set, and as a result the joint \( P \) value increases to .042. None of the following 44 ranked SNPs replicated in the additional samples genotyped.

Fine mapping of RFC1 did not provide additional insights: significance levels are comparable to the original significance observed for rs2066786 and can be explained by the linkage disequilibrium structure in the region (Supplementary figure 2).

Effect of Rare Coding Nonsynonymous Variants

The rare coding nonsynonymous variants uncovered by sequencing RFC1, SCN1A, and ANPEP were genotyped. No significant associations (Fisher exact test) were found between these rare mutations and the case/control status (data not shown). Collapsing the counts of carriers of rare variants across SNPs within genes did not provide additional insights.

Lack of Replication of Associations Reported in the Literature

An association between symptomatic WNV disease and homozygosity for the CCR5Δ32 mutation in the chemokine receptor gene CCR5 was initially reported [16, 17]. More recently, this association was not replicated, but results suggestive of a link to clinical manifestations of infection with CCR5Δ32...
Table 2. Results From the Primary Association Analyses Between Single-Nucleotide Polymorphisms (SNPs) and Risk of Neuroinvasive Disease in Patients Infected With the West Nile Virus

| SNP       | Rank | Chromosome | Position | Allele | Frequency |
|-----------|------|------------|----------|--------|-----------|
| rs2066786<sup>a</sup> | 1    | 4          | 38978423 | T      | 0.3811   |
| rs2298771<sup>b</sup> | 2    | 2          | 166601033 | T      | 0.3911   |
| rs25651<sup>a</sup> | 3    | 15         | 88136791 | T      | 0.2857   |
| rs11575302<sup>b</sup> | 5    | 7          | 50657187 | A      | 0.1997   |
| rs2177396<sup>b</sup> | 8    | 3          | 197001272 | T      | 0.1462   |
| rs7163367<sup>a</sup> | 10   | 15         | 88061148 | A      | 0.3962   |
| rs10839601<sup>b</sup> | 12   | 11         | 6696711 | T      | 0.1821   |
| rs3738573<sup>a</sup> | 17   | 1          | 84638683 | C      | 0.3222   |
| rs3739990<sup>c</sup> | 18   | 5          | 78457714 | A      | 0.2637   |
| rs323347<sup>a</sup> | 19   | 8          | 30825765 | G      | 0.1572   |
| rs11652709<sup>f</sup> | 22   | 17         | 53626092 | C      | 0.3527   |
| rs1805073<sup>b</sup> | 23   | 5          | 78362505 | T      | 0.2597   |
| rs2824271<sup>b</sup> | 25   | 21         | 18591988 | G      | 0.2071   |
| rs1058587<sup>b</sup> | 26   | 19         | 18360421 | G      | 0.2817   |
| rs10778292<sup>a</sup> | 27   | 12         | 10278441 | C      | 0.1177   |
| rs5370<sup>b</sup> | 29   | 6          | 12404240 | T      | 0.2429   |
| rs3816988<sup>b</sup> | 30   | 15         | 60898791 | T      | 0.1986   |
| rs12371985<sup>b</sup> | 32   | 12         | 93466790 | G      | 0.0544   |
| rs228777<sup>b</sup> | 33   | 5          | 150900300 | A      | 0.0546   |
| rs794999<sup>b</sup> | 34   | 3          | 12204014 | G      | 0.2829   |
| rs16826068<sup>b</sup> | 35   | 1          | 39669641 | G      | 0.2587   |
| rs2278428<sup>b</sup> | 36   | 19         | 60109865 | C      | 0.0607   |
| rs2161488<sup>b</sup> | 38   | 19         | 9949270 | G      | 0.4482   |
| rs2270962<sup>b</sup> | 39   | 10         | 102060033 | T      | 0.0571   |
| rs2270915<sup>b</sup> | 40   | 5          | 32822145 | A      | 0.2397   |
| rs4432013<sup>b</sup> | 41   | 11         | 4687755 | G      | 0.1911   |
| rs1527014<sup>b</sup> | 42   | 12         | 16288943 | G      | 0.0383   |
| rs13095016<sup>b</sup> | 43   | 3          | 196999088 | G    | 0.1434   |
| rs11079339<sup>b</sup> | 44   | 17         | 53625440 | A      | 0.1607   |
| rs2236358<sup>b</sup> | 47   | 1          | 226002384 | A    | 0.0546   |
| rs6293<sup>d</sup> | 48   | 7          | 50563293 | C      | 0.0102   |
| rs2523421<sup>i</sup> | 51   | 6          | 29488418 | G      | 0.1146   |
| rs11076256<sup>a</sup> | 53   | 16         | 57309966 | T      | 0.0973   |
| rs1128349<sup>i</sup> | 54   | 7          | 72735589 | T      | 0.4304   |
| rs7574414<sup>d</sup> | 55   | 2          | 227952184 | A    | 0.1518   |
| rs2287939<sup>i</sup> | 57   | 5          | 34034639 | A      | 0.2577   |
| rs2299102<sup>i</sup> | 59   | 16         | 65171508 | C      | 0.0964   |
| rs7143633<sup>i</sup> | 61   | 14         | 20619732 | G      | 0.1614   |
| rs3754279<sup>i</sup> | 63   | 3          | 84640197 | A      | 0.2711   |
| rs7591849<sup>i</sup> | 64   | 2          | 158821226 | G    | 0.4677   |
| rs11540407<sup>i</sup> | 65   | 12         | 7148684 | C      | 0.2308   |
| rs980006<sup>i</sup> | 66   | 6          | 27764477 | C      | 0.1481   |
| rs1047891<sup>i</sup> | 75   | 1          | 211097780 | C     | 0.4768   |
| rs2241988<sup>i</sup> | 89   | 3          | 57517212 | T      | 0.4768   |
| rs6046<sup>c</sup> | 132  | 13         | 112821159 | A   | 0.1027   |
| rs2240154<sup>i</sup> | 135  | 19         | 954171 | T      | 0.1759   |
| rs5748648<sup>i</sup> | 259  | 22         | 15660821 | A      | 0.0428   |

SNPs were selected to undergo replication as detailed below. Abbreviations: A, adenine; C, cytosine; CI, confidence interval; G, guanine; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; T, thymine.

<sup>a</sup> Selected for replication according to results from 445 case patients and 813 control patients.
<sup>b</sup> Selected for replication according to results from the complete sample of case patients and control patients.
<sup>c</sup> Selected for replication according to results from the complete sample of case patients and control patients but replaced with rs10839601 in the replication panels. Frequency estimates and ORs (along with their 95% CIs) are calculated with respect to allele 1. Also included are significance levels for tests of HWE.
mutation were reported [18]. The plausibility of a susceptibility locus in the OAS gene cluster was reported [19, 20]. More recently, a candidate gene study examining the OAS gene cluster alone suggested a predisposition to WNV infection [21]. There were 13 (1.58%) of 821 case patients who were homozygous for the CCR5Δ32 deletion, compared with 13 (1.05%) of 1233 control patients (OR, 1.51 [95% CI, .70–3.27]; P = .30). For genes in the OAS cluster, ORs ranged from 1.1 to 1.13 for 4 OAS variants with all P values >.05 (Supplementary table 2).

**Power to Detect an Association**

We calculated the power that a sample consisting of 824 case patients and 1246 control patients (the sample used in the joint analysis) has to detect an association with a polymorphism whose risk is assumed to act additively with the number of risk alleles an individual possesses assuming homogeneity across collection centers. Supplementary figure 3 shows that the sample has 80% power to detect an association at P < 4.7 × 10⁻⁶ (a level deemed to be significant even after a Bonferroni correction for all SNPs tested) for relative risks as low as 1.5 (depending on the risk allele frequency).

**DISCUSSION**

We sought to test for common genetic polymorphisms associated with severe WNV complications in a North American population using mainly nonsynonymous SNPs. We identified a variant in each of 3 genes (RFC1, SCN1A, ANPEP) potentially implicated in susceptibility to such neurological complications. Joint analysis showed for RFC1-rs2066786, P = .0022; SCN1A-rs2298771, P = .005 and ANPEP-rs25651, P = .042. SNPs in other candidate genes (OAS, TLR3 [Toll-like receptor 3 gene]) were not significant. Our findings reveal preliminary information that genetic variants may play a role in susceptibility to severe WNV disease.

Human replication factor C (RFC), also called activator-1, is a multimeric primer-recognition protein consisting of 5 distinct subunits [27]. Human RFC was purified from extracts of HeLa cells as a host factor essential for the in vitro replication of simian virus 40 DNA. RFC allows for efficient elongation of DNA in the presence of human single-stranded DNA binding protein. Using interaction cloning, Uchiumi et al found that the large subunit of RFC interacts with the DNA sequence repeats of telomeres [30]. They found that RFC recognizes the 5' -phosphate termini of double-stranded telomeric repeats. The authors suggested that RFC may be involved in telomere stability or turnover. Presumably, if RFC1 plays a pathogenic role in WNV, it could be with respect to facilitating viral replication.

To explain the lack of replication of rs2066786, we assessed the effect of this variant on the 3 subcategories of neuroinvasive disease (meningitis, encephalitis, or acute flaccid paralysis). As shown in Table 2, there was no difference in effects. Population structure in the replication cohort is another possible explanation for lack of replication. Indeed, continental differences in European ancestry have been described [31]. However, we did not identify any differences even adjusting for ancestry informative markers. We reviewed phenotype data and could not identify any systematic differences in phenotyping.

An association between the 32-bp deletion in CCR5 and West Nile clinical symptoms has been reported [18]. We found no evidence of any effect. One possible reason for the discrepancy is that we compared case patients with control patients, all of whom were symptomatic when infected with WNV. In contrast, the published reports compared case patients with control patients with no symptoms. Notably, we found no association between any of the OAS polymorphisms and WNV. We believe that this highlights the importance of conducting human population-based studies. Although TLR was also reported to be a mechanism in a murine model, we saw no significant effect in this population-based study [32].

Strengths of this study include the rigorous definitions and procedures used to confirm case and control status. All clinical and laboratory data had to have supportive documentation, including laboratory records and clinical information. Selection of case patients and control patients was as unbiased as could be achieved. In the states and provinces that we selected, all of those who were infected were invited to participate through notification from public health laboratories where the great majority of testing occurred. We acknowledge that sample size for such an association study was limited, but this is a function of the number of WNV neuroinvasive cases seen in North America.

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