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BRIEF REPORT

TLR3 Mutations in Adult Patients With Herpes Simplex Virus and Varicella-Zoster Virus Encephalitis

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Defects in genes of the Toll-like receptor 3 (TLR3) pathway are associated with susceptibility to herpes simplex virus type 1 encephalitis (HSE). We analyzed a cohort of 11 adult Italian patients in whom viral encephalitis developed. We detected 2 rare missense mutations in *TLR3*: 1 in a patient with HSE (p.Leu297Val) and 1 in a patient with varicella-zoster virus encephalitis (p.Leu199Phe). Both mutations are extremely rare in human populations and have pathogenicity scores highly suggestive of a functional effect. Data herein expand the phenotypic spectrum of *TLR3* mutations to varicella-zoster virus encephalitis and support the role of *TLR3* genetic defects as risk factors for HSE in adults.

Keywords. herpes simplex virus encephalitis; varicella-zoster virus encephalitis; *TLR3*; adult patients.

Herpes simplex virus (HSV) and varicella-zoster virus (VZV) are enveloped double-strand DNA viruses belonging to the alphaherpesvirus subfamily. Both viruses are neurotropic and establish persistent infections in humans [1]. The most serious complication associated with HSV and VZV infection is encephalitis [2]. HSV type 1 (HSV-1) encephalitis (HSE) is the most common cause of nonepidemic acute encephalitis in Western countries [2]; VZV is the second most common infectious cause across all age groups [2].

Genetic screenings in children with HSE indicated that mutations in the Toll-like receptor 3 (TLR3) gene (*TLR3*) [3–5] or in other genes participating in the TLR3 pathway (*TLR3, TICAM1, TRAF3, UNC93B1, TBK1*, and *IRF3*) represent risk factors for HSE [6]. Because children with genetic defects in the TLR3 pathway do not have increased susceptibility to other pathogens, a specific role of these genes for HSE risk was suggested [7]. Whole-exome screening of adult patients with HSE detected rare variants in genes of the TLR3 pathway (*TLR3, TICAM1*, and *IRF3*) [8].

TLR3 localizes to the endosomes and recognizes double-strand RNA, an almost-universal intermediate of viral replication. Its expression in the brain and in peripheral nerves [9, 10] indicates that TLR3 plays an important role in controlling viral spread in the central nervous system (CNS). Thus, mutations in genes participating in the TLR3 pathway may predispose to encephalitis caused by neurotropic viruses other than HSV-1, including VZV.

METHODS

Patient Cohort

All consecutive adults patients (>18 years old) admitted to the San Gerardo Hospital, Monza, Italy, with suspected or microbiologically confirmed viral meningitis and/or viral encephalitis between January 2008 and December 2012 were considered for enrollment in the study. Diagnosis of viral encephalitis/meningitis was made according to clinical criteria (fever, meningeal signs, altered consciousness, and focal neurologic deficits) associated with typical cerebrospinal fluid findings, particularly mildly increased white blood cell count, mainly lymphocytes (probably <250/μL), mildly elevated protein concentration (usually <150 mg/dL), and normal glucose concentration in the absence of bacterial growth.

A total of 11 patients were enrolled. In a subset of patients, the etiologic diagnosis of meningitis/encephalitis was confirmed through molecular identification of the virus in cerebrospinal fluid (confirmed viral encephalitis/meningitis). Specifically, 3 patients tested positive for HSV-1, and 2 for VZV. In the remaining cases, no etiologic diagnosis was made (suspected viral encephalitis/meningitis). In the HSE cases, HSV-1 serology (immunoglobulin M or G) was not performed because diagnosis of either primary or recurrent HSV-1 infection would not change the treatment protocol. Of the 2 patients with VZV encephalitis, 1 had a history of shingles during childhood (patient 2 in Table 1), and in the other herpes zoster developed approximately 1 month before the onset of neurologic symptoms. Thus, encephalitis can be attributed to viral reactivation in these 2 subjects.

A detailed description of the clinical characteristics of the 3 patients with *TLR3* mutations is provided in the Supplementary Data. Written informed consent was obtained from all subjects. The study was reviewed and approved by the institutional review board of the IRCCS E. Medea, Bosisio Parini, Italy (No. 072/12-CE; 6 May 2012).

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Table 1. Demographic, Clinical, and Genetic Characteristics in Patients with *TLR3* Mutations

Characteristic	Patient 1	Patient 2	Patient 3
Sex	Female	Male	Male
Age, y	49	31	69
TLR3 mutation	p.Leu297Val	p.Leu199Phe	c.−8 + 6T > C
Symptoms	Fever, confusion, impaired level of consciousness	Headache, lower back pain, photophobia, nausea and vomiting, stiff neck, and positive Lasègue sign	Confusion, dysarthria, impaired level of consciousness, and seizures
CSF findings	Mononuclear cell count, 230/μL; protein, 90 mg/dL; glucose, 82 mg/dL	Mononuclear cell count, 8/μL; protein, 132 mg/dL; glucose, 54 mg/dL	Mononuclear cell count 80/μL; protein, 52 mg/dL; glucose, 109 mg/dL
PCR (of CSF samples)	HSV-1 DNA positive	VZV DNA positive	HSV-1 DNA positive
MR imaging	Hyperintense altered signal (T2) in right temporal lobe, hippocampus, amygdala, and insular cortex; partially altered signal in left temporal lobe; cortical edema	Slight hyperintensities in right primary motor cortex	Encephalitic changes in left temporal lobe, insular cortex, and left thalamus
Treatment	Acyclovir (4 wk)	Acyclovir (3 wk) and oral valacyclovir (1 wk)	Acyclovir (33 d)
Outcome (at discharge)	Complete recovery with residual vacuous behavior	Complete recovery	Poor cognitive abilities and motor difficulties

Abbreviations: CSF, cerebrospinal fluid; HSV-1, herpes simplex virus type 1; MR, magnetic resonance; PCR, polymerase chain reaction; *TLR3*, Toll-like receptor 3 gene; VZV, varicella-zoster virus.

Genetic Analysis

Genomic DNA was extracted from peripheral blood. All exons of *TLR3*, *TICAM1*, and *IRF3* were amplified by polymerase chain reaction. Primer sequences were located in the flanking intronic sequences so as to allow analysis of splicing junctions. Polymerase chain reaction products were treated with ExoSAP-IT, directly sequenced on both strands with a Big Dye Terminator sequencing Kit, and analyzed with an Applied Biosystems ABI 3130 XL Genetic Analyzer. Sequences were assembled using AutoAssembler software (Applied Biosystems), version 1.4.0, and inspected manually by 2 distinct operators.

In Silico Analysis and 3-Dimensional Structures

REVEL (Rare Exome Variant Ensemble Learner) scores for all possible missense substitutions in human *TLR3* (n = 6037) were downloaded from the dedicated Web site (https://sites.google.com/site/revelgenomics/) [11]. CADD (Combined Annotation Dependent Depletion) scores were calculated through the "score variants" utility of the CADD server (version 1.3; http://cadd.gs.washington.edu/) [12]. MetaSVM and MetaLR scores were retrieved through ANNOVAR (Annotate Variation) [13, 14].

The protein 3-dimensional structure of mouse TLR3 in complex with double-strand DNA was derived from the Protein DataBank (Protein DataBank identifier, 3CIY). Mutations were mapped onto the structure using PyMOL software (PyMOL Molecular Graphics System, version 1.5.0.2; Schrödinger).

RESULTS

Identification of *TLR3* Mutations in 3 Patients With Herpesvirus Encephalitis

We determined the sequence of all exons and flanking splicing junctions of *TLR3*, *TICAM1*, and *IRF3* in 11 adult patients with confirmed or suspected viral encephalitis. We focused on this gene subset because mutations were previously associated with HSE in adults [8]. No mutation was detected in *TICAM1* and *IRF3*, whereas 2 rare missense variants and a rare intronic mutation were detected in *TLR3* (Table 1).

Specifically, the p.Leu297Val mutation, previously described in a Danish adult patient with HSE [8], was identified in a 59-year-old women with diagnosed HSE who fully recovered after 4 weeks of antiviral treatment (patient 1 in Table 1). The second missense mutation, p.Leu199Phe, was detected in a 32-year-old man with meningoencephalitis after VZV reactivation (patient 2 in Table 1). The intronic mutation (c.−8 + 6T > C, rs765240183), harbored by a patient in whom HSE developed (patient 3 in Table 1), was located 6 nucleotides downstream from the donor splice site of the first noncoding exon.

The p.Leu297Val variant was previously reported in the dbSNP database (rs35311343); the frequency of the Val allele in the ExAc database is 0.0015 (calculated on 121 298 chromosomes). The other missense variant (p.Leu199Phe; dbSNP, rs753482575) has an allele frequency in the ExAc database of 0.0000086 (116 438 chromosomes). Finally, the intronic variant (c.−8 + 6T > C) is recorded in the dbSNP database (rs765240183) with no reported frequency, and it is not present in the 1000 Genomes database. The intronic position is not covered by the data in the ExAc database.

Pathogenicity Scores and Structural Mapping

We next investigated the potential pathogenicity of the identified variants compared with previously described *TLR3* mutations observed in patients with HSE. The recently developed "ensemble" scores REVEL and MetaSVM/MetaLR were shown to display very high sensitivity and specificity [11, 13]. These methods combine pathogenicity predictions from several individual features [11, 13]. In particular, REVEL scores perform very well in distinguishing pathogenic from neutral variants with low allele frequencies (<0.5%) [11].

REVEL scores >0.4 discriminate pathogenic changes with a specificity >0.8 (the sensitivity is similarly about 0.8 for this value). For MetaSVM, a score of 0 is taken as a cutoff to classify mutations as deleterious (the cutoff is 0.5 for MetaLR). MetaSVM has a true-positive rate of 0.80–0.87 and a false-negative rate of 0.13–0.21 [13].

We obtained REVEL and MetaSVM scores for all possible missense substitutions in human *TLR3* (Figure 1A). The 2 scores showed good correlation (Spearman rank correlation coefficient, 0.74; $P < 10^{-15}$) and most *TLR3* missense substitutions displayed low REVEL and MetaSVM values (Figure 1A).

The p.Leu199Phe and p.Leu297Val mutations, as well as the p.Leu360Pro change described elsewhere [3], had REVEL scores >0.4 and were predicted as deleterious by MetaSVM. MetaLR predicted as pathogenic p.Leu199Phe and p.Leu360Pro, with p.Leu297Val very close to the cutoff (Figure 1A). REVEL scores were also high for 4 *TLR3* mutations previously identified in children with HSE and shown to affect receptor activity in fibroblasts in vitro [3–5]. However, these mutations were not predicted to be deleterious by MetaSVM or MetaLR (Figure 1A), thus representing false-negative predictions for these methods.

Finally, p.Leu199Phe and p.Leu297Val, as well as previously described HSE mutations with functional effect, had high scores (>20) for CADD, another ensemble prediction method [12]. A table reporting values for individual REVEL predictive features is available as Supplementary Table 1.

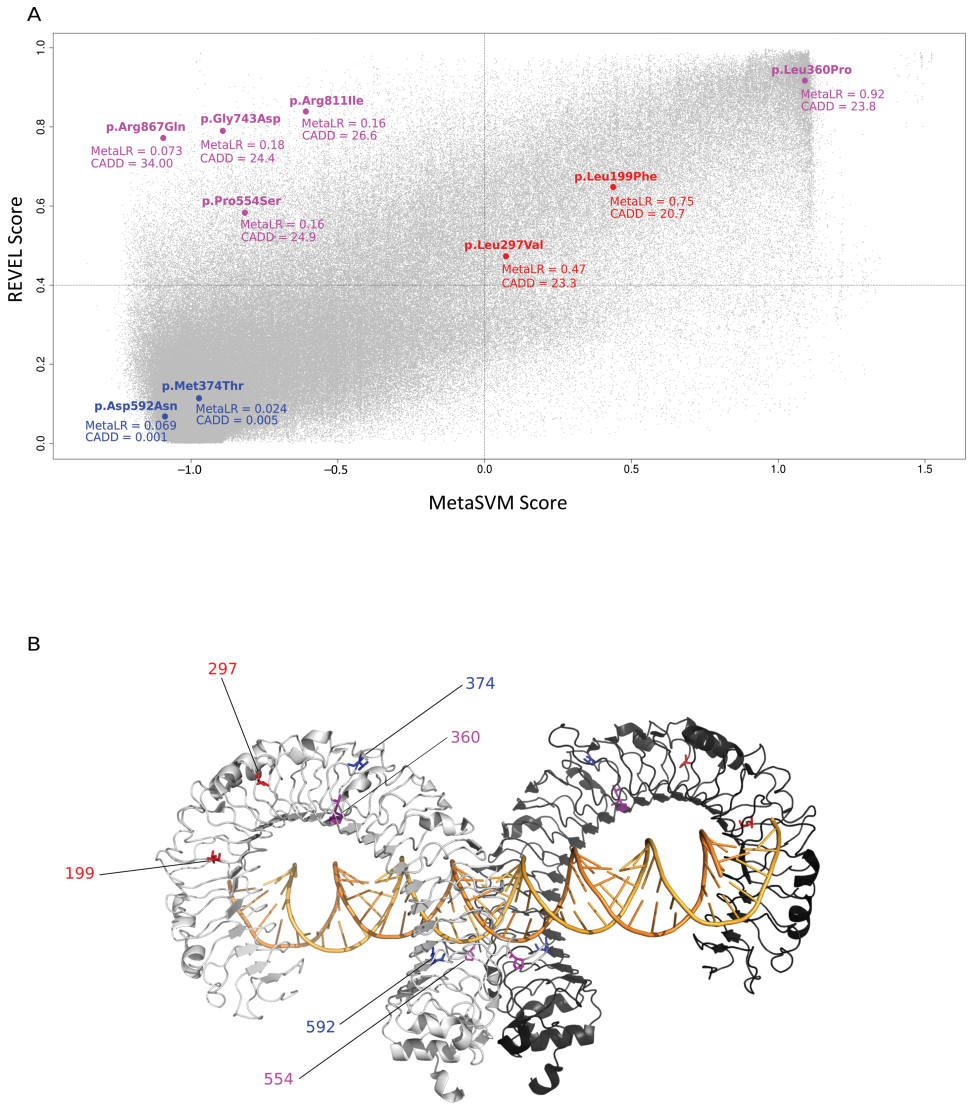

Figure 1. Pathogenicity scores and structural mapping of Toll-like receptor 3 (TLR3) gene mutations. *A,* Distribution of REVEL (Rare Exome Variant Ensemble Learner) and MetaSVM scores for all possible missense mutations in human *TLR3.* Hatched horizontal line denotes a REVEL score of 0.4 (corresponding to a specificity of 0.8); hatched vertical line, a metaSVM score of 0 (cutoff to classify a mutation as deleterious). Values corresponding to the mutations we identified are shown in red. Scores of previously described *TLR3* mutations detected in children with herpes simplex virus type 1 encephalitis are shown in magenta, together with scores for 2 benign variants in blue. For each mutation, CADD (Combined Annotation Dependent Depletion) and MetaLR scores are also reported. *B, TLR3* mutations are mapped onto the 3-dimensional structure of the mouse TLR3 dimer in complex with double-strand DNA. Mutations are labeled on a single monomer only. Color codes are as in *A.*

Importantly, 2 amino acid replacement that have no effect on TLR3 responsiveness in fibroblasts [3] showed very low scores with all prediction methods (Figure 1A). Overall, these data suggest that p.Leu199Phe and p.Leu297Val are pathogenic. Based on the results for known pathogenic *TLR3* variants with experimentally verified functional effect, REVEL and CADD scores display better performances than MetaLR/MetaSVM scores.

REVEL and MetaSVM/MetaLR scores could not be calculated for the rs765240183 intronic variant, because they were developed to evaluate missense substitutions. We thus calculated the CADD score for rs765240183, which was low (1.67), suggesting that the variant is not pathogenic [12]. We next mapped missense *TLR3* mutations detected onto the 3-dimensional structure of the ectodomain. Neither the mutations we detected nor those previously described in children with HSE are directly involved in double-strand DNA binding (Figure 1B).

DISCUSSION

We report heterozygous missense mutations in *TLR3* in 2 patients with HSE or VZV encephalitis. One of the 2 variants, p.Leu297Val (patient 1, with HSE), was previously described in a Danish adult patient with HSE. Because the 297Val allele is definitely uncommon, our data strongly support its causal association with HSE susceptibility. The other variant, p.Leu199Phe (patient 2, with VZV encephalitis), has never been reported in patients with HSE, and, to our knowledge, no *TLR3* mutation screening has ever been performed in patients with VZV encephalitis. The 199Phe allele is extremely rare in humans (detected in 1 of >100 000 chromosomes) and, as is the case with p.Leu297Val substitution, has pathogenicity scores higher than or comparable to those of previously characterized HSE mutations that impair TLR3 signaling. Overall, the combined analysis of several prediction methods supported the causal role of p.Leu199Phe and p.Leu297Val in the susceptibility to herpesvirus encephalitis. We stress, however, that a definitive assessment of the functional effect of these variants will require experimental analysis.

A previous analysis of the functional role of p.Leu297Val was performed on peripheral blood mononuclear cells and revealed no defective response to polyinosinic-polycytidylic acid (poly I:C) or HSV-1 [8]. However, several cell types, including leukocytes, from patients with HSE and *TLR3* mutations display normal responses to agonists and HSV-1 challenge. Indeed, TLR3 function seems to be specifically important in the CNS [4, 5].

Both HSV and VZV are neurotropic and establish latent infection in the trigeminal and other ganglia. Although the mechanisms of viral access to the brain are not fully determined, spread via the trigeminal and olfactory nerves seems to be responsible for CNS infection with HSV and VZV [1]. Because TLR3 is constitutively expressed in Schwann cells [10], genetic defects of this receptor (or molecules in the downstream signaling cascade) may confer specific susceptibility to both HSV- and VZV-mediated CNS disease.

Genetic defects in the TLR3 pathway were initially identified in pediatric HSE cohorts [6]. A 2015 study, however, described such mutations in adults [8]. Based on data from 16 patients, the authors suggested that genetic defects in innate immunity genes may underlie HSE susceptibility in 62% of adult cases, whereas the proportion seems to be much smaller in children [3, 8, 15]. Our data support the conclusion that the genetic basis for the susceptibility to herpesvirus encephalitis is similar in children and adults and that mutations in adult patients are relatively common.

Most previously identified *TLR3* mutations associated with HSE were heterozygous missense changes [3, 4, 8]. Several missense mutations in *TLR3* have a dominant negative effect on TLR3 signaling, possibly owing to the multimeric nature of active TLR3 signaling complexes [3, 4]. It is thus conceivable that both p.Leu297Val and p.Leu199Phe confer autosomal dominant susceptibility to herpesvirus encephalitis.

Clearly, our study has several limitations. The sample size was small, and the etiologic diagnosis of viral encephalitis was reached for a subset of patients. Inference as to the pathogenicity potential of the identified mutations relies on bioinformatic predictions, and experimental validation will be required to conclusively associate these mutations to the risk of herpesvirus encephalitis. However, our data support the role of *TLR3* mutations as risk factors for HSE in adults and warrant screening for such mutations in patients with VZV encephalitis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Note

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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