Search for viral infections in cerebrospinal fluid from patients with autoimmune encephalitis

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Major article summary:
Autoimmune encephalitis was diagnosed in 8 (4%) out of 200 consecutive hospitalized patients with encephalitis. Metagenomics and RT-PCR/PCR detected the presence of a eukaryotic virus (HSV, TTV and representative of Enterovirus genus) in cerebrospinal fluid from three autoimmune encephalitis patients.

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ABSTRACT

BACKGROUND

It has been reported that a virus-mediated brain tissue damage can lead to autoimmune encephalitis (AE) characterized by the presence of antibodies against neuronal surface antigens. In the study we investigate the presence of viruses in cerebrospinal fluid (CSF) from patients with AE using RT-PCR/PCR and shotgun metagenomics.

METHODS

CSF samples collected from two hundred patients with encephalitis were tested for the presence of antibodies against anti-glutamate receptor (NMDAR), contactin-associated protein 2 (CASPR2), glutamate receptors (type AMPA1/2), leucine-rich glioma-inactivated protein 1 (LGI1), dipeptidyl aminopeptidase-like protein 6 (DPPX) and GABA B receptor and those found positive were further analyzed with real-time RT-PCR/PCR for common viral neuroinfections and shotgun DNA- and RNA-based metagenomics.

RESULTS

Autoantibodies against neuronal cells were detected in CSF from 8 individuals (4% of all encephalitis patients): 7 (3.5%) had anti-NMDAR and one (0.5%) had anti-GABA B. RT-PCR/PCR identified human herpes virus type 1 (HSV-1); (300 copies/ml) and the representative of Enterovirus genus (550 copies/ml) in one patient each. Torque teno virus (TTV) was found in another patient using metagenomic analysis and its presence was confirmed by specific PCR.

CONCLUSIONS

We detected the presence of HSV, TTV and Enterovirus genus in CSF samples from 3 out of 8 AE patients. These findings support the concept of viral involvement in the pathogenesis of this disease.

Key words: autoimmune encephalitis; anti-NMDAR; metagenomics; virus; NGS
INTRODUCTION

Autoimmune encephalitis (AE), which is characterized by the presence of antineuronal or anti-glia antibodies, was reported to constitute 4.2% and 7.9% of all encephalitis cases in the US and the United Kingdom, respectively [1, 2]. However, some studies reported markedly higher percentage [2, 3] particularly among pediatric patients [4]. Autoantibodies in AE are classified into two main categories: antibodies against neuronal cells surface and against intracellular antigens [5]. While autoimmune reactions against intracellular antigens are linked with irreversible outcomes and limited response to therapy, patients with surface autoantibodies have a more favorable prognosis and respond well to immunosuppressive treatment [6, 7]. Intracellular antibodies are almost invariably part of paraneoplastic syndrome but only a minority of patients with autoantibodies against surface antigens have concurrent tumor [8-10]. There is mounting evidence that in contrast to intracellular antibodies, autoantibodies against surface antigens such as anti-N-Methyl-d-aspartate receptor (anti-NMDAR) and anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (anti-AMPAR) are directly pathogenic [11-14].

It is currently unclear what causes activation and migration of autoantibody-producing B lymphocytes across the blood–brain barrier [15]. It has been proposed that a virus-mediated brain tissue damage could lead to exposure of the normally sequestered neuronal cell antigens or the cause of autoantibodies production could be “molecular mimicry” of viral proteins [16-18]. The primary candidate is Herpes simplex virus (HSV) which was reported to be capable of triggering autoimmunity to NMDAR, dopamine D2 receptor (D2R) and γ-aminobutyric acid type A receptor (GABA-AR) [18, 19]. However, a variety of other viral agents including West Nile virus (WNV), Varicella zoster virus (VZV)
and even Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were associated with the development of AE [20-23].

In the present study we used real-time RT-PCR/PCR and next-generation sequencing (NGS) metagenomics to search for the presence of viruses in cerebrospinal fluid (CSF) from patients with AE.

METHODS

PATIENTS

The analysis was conducted on 200 consecutive patients who were part of a large ongoing single-center study on the etiology of encephalitis in Poland. Details of the study, including criteria for inclusion and data on the first 96 patients were published previously [24]. CSF and serum were collected at the time of hospital admission. CSF was centrifuged at 1.200 rpm for 20 min at 4°C, and kept frozen -80°C.

AUTOANTIBODY DETECTION

The presence of antibodies against NMDAR, contactin-associated protein 2 (CASPR2), AMPAR1/2, leucine-rich glioma-inactivated protein 1 (LGII1), dipeptidyl aminopeptidase-like protein 6 (DPPX) and GABA B receptor was evaluated using commercially available indirect immunofluorescence test (Autoimmune Encephalitis Mosaic 6; Euroimmune, Germany). Fluorescence was read with Nikon Eclipse 80i (Nikon, Japan) microscope at ×20 and ×40 magnifications. CSF samples from all patients were tested regardless whether any etiological factor of encephalitis was identified or not. All positive results were confirmed in an independent run and read by a technician experienced in routine diagnostics of autoimmune encephalitis antibodies.
VIRUS-SPECIFIC RT-PCR/PCR

CSF samples were analyzed with in-house quantitative real-time RT-PCR/PCR assays described previously [25-28] using RNA and DNA extracted from 200 µl of CSF by Trizol LS (Thermo Fisher Scientific, USA) and NucleoSpin Plasma XS kit (Macherey Nagel, Germany), respectively. The following pathogens were searched for: herpes simplex viruses type 1/2 (HSV-1/2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), human adenoviruses (HAdVs) and enteroviruses (EV: Coxsackie A9, A16, B2, B3, B4, B5; ECHO 5, 6, 9, 11, 18, 30 and enterovirus 71). Limits of detection (LOD) for quantitative PCRs were as follows: for HSV-1 - 253 viral copies/ml, HSV-2 - 369 viral copies/ml, VZV - 150 viral copies/ml, CMV – 403 viral copies/ml, EBV - 226 viral copies/ml, HHV-6 - 111 viral copies/ml, HHV-7 – 153 viral copies/ml, HAdV - 102 viral copies/ml and EV - 240 viral copies/ml.

SEROLOGICAL TESTING

Routine serological testing included anti-HSV-1/2 IgG/IgM, anti-VZV IgG/IgM and anti-TBEV IgG/IgM, (Institut Virion/Serion GmbH, Germany). Tests were performed and interpreted following manufacturer’s recommendations.

VIRAL METAGENOMICS

225 µl of each CSF were filtrated using Millex-HV Syringe Filter Unit (Merck KgaA, Germany) with a pore size of 0.45 µm and digested with 2U of TURBO DNase (Thermo Fisher Scientific, USA) for 30 min. Next, filtrated and digested CSF samples were subjected to RNA extraction with TRIzol LS (Thermo Fisher Scientific, USA) or DNA extraction using NucleoSpin Plasma XS kit (Macherey-Nagel, Germany). RNA and DNA were eluted in 5 µl and 12 µl of water, respectively. Due to typical low yield of DNA/RNA extraction from CSF a preamplification step was introduced to generate NGS libraries for sequencing. In short, five microliters of RNA was first reversely transcribed for 5 min at 65°C and preamplified by
a single-primer isothermal amplification (Ribo-SPIA) using Ovation RNA-Seq V2 system (NuGEN, San Carlos, USA). For DNA, the whole amount was preamplified with SeqPlex Enhanced DNA Amplification kit (Sigma-Aldrich, USA). Preamplified cDNA/DNA was purified using 0.8 ratio of Agencourt AMPure XP beads (Beckman Coulter, USA) to reaction mixture and eluted in 30 μl of water. Libraries for sequencing were prepared by Nextera XT Kit (Illumina, USA) using one ng of preamplified cDNA/DNA and following manufacturer’s protocol with small modifications: amplification was performed with 14 cycles instead of 12 and the ratio of Agencourt AMPure XP beads (Beckman Coulter, USA) added to reaction mixture was 0.6. The quality and average length of NGS libraries were assessed using Bioanalyzer (Agilent Technologies, USA) and DNA HS kit (Agilent Technologies, USA). Finally, the samples were indexed, pooled and sequenced on Illumina HiSeq (101nt, paired-end reads).

NGS reads were evaluated for quality using FastQC software [29] and those with Phred quality score over 30 were trimmed with Trimmomatic [30] and mapped to human genome using Stampy [31]. Next, all non-human reads were aligned by Bowtie2 [32] to database containing complete viral genomes obtained from NCBI Reference Sequence Database (RefSeq). Reads matching viral genomes were sorted and counted with SAMtools [33] and phyloseq [34] package in R. Visualization of alignments and coverage were analyzed using CLC Genomics Workbench (Qiagen, USA).

The following criteria were applied for positive virus detection by metagenomic analysis: i) a minimum of three reads specific for a particular viral species had to be present ii) specific reads had to be distributed over the genome. Similar criteria were previously used by other groups for viral identification by NGS [35, 36].
PATIENT CONSENT STATEMENT

All patients gave a written informed consent and all research was performed in accordance with the relevant guidelines and regulations. The study was approved by the Internal Review Board of the Medical University of Warsaw, Poland.

RESULTS

Autoantibodies against neuronal cells were detected in 8 individuals (4% of all encephalitis patients): 7 (3.5%) had anti-NMDAR and one (0.5%) had anti-GABA B. Only one of these patients had anti-NMDAR in serum (Table 1). All these samples were tested at serial dilutions $10^1$ - $10^4$ and deemed to be low positive (1:10). None of the patients had teratoma or any other type of cancer.

The age of autoantibodies-positive patients ranged from 20 to 62 years and the majority were female (62.5%). Seizures were the most prominent clinical manifestation as they were present in almost every patient and four patients had psychotic symptoms. Some demographic and clinical data on these patients are presented in Table 1.

All patients positive for autoantibodies were further analyzed for the presence of common neurotropic viruses in CSF using quantitative in-house RT-PCR/PCR and for the presence of specific anti-viral antibodies using commercial assays (Table 1). One patient was found to be infected with HSV-1 (viral load 300 copies/ml). Another patient was found to be infected by an enterovirus (our quantitative RT-PCR would detect several enteroviruses but did not discriminate between species) and the estimated viral load was 550 copies/ml.
Detailed data on metagenomic analysis and corresponding PCR and serological test results are shown in Table 1. Our metagenomic analysis consisted of two-pronged approach based on separate RNA- and DNA-preamplification. Numbers of raw reads per sample for DNA and RNA analysis were 12,893,313 and 14,469,597, respectively. After quality checking and trimming the average number of filtered reads for RNA and DNA analysis was 12,653,056 and 14,137,536, respectively. The number of reads aligning to viral genomes ranged from 1,015 to 132,884 (median: 6,645) and was similar for RNA and DNA analysis (median: 6,493 vs 6,645) Only one eukaryotic virus - Torque teno virus (TTV) – was identified (Table 1). The presence of TTV-DNA was confirmed by specific PCR detecting viral non-coding region using primer NG133 (91-115 nucleotide position based on GenBank AB017610.1) and NG147 (position 211-233) and following procedure described by Okamoto et al [37].

Although metagenomics identified eukaryotic viral agent in only one CSF sample, viral reads were detected in all. The vast majority of these viruses were bacteriophages, whereas the remaining viral reads either did not fulfil the criteria for positivity or were classified as contaminants or artifacts (not shown).
DISCUSSION

In the current study anti-neuronal autoantibodies were detected in 8 (4%) out of 200 patients with clinical diagnosis of encephalitis, which is similar to the proportion reported for the USA (4.2%; based on anti-NMDAR) [38] and only slightly lower than the one reported for Hungary (5.8%; based on anti-NMDAR, anti-LGI1, anti-Caspr2, anti-GABABR, anti-AMPAR1, and anti-AMPAR2) [39] and UK (7.9%; based on anti-VGKC, anti-NMDAR) [40]. In our study the vast majority of detected autoantibodies were anti-NMDR (87.5%) which is compatible with other reports; e.g. in the study by Saraya et. al. [41] anti-NMDAR antibodies were present in 81% of AE cases with detectable neuronal surface antibodies.

However, our study could have underestimated the prevalence of AE as patients were required to have increased CSF pleocytosis and/or elevated protein to be diagnosed with encephalitis and some AE cases have been observed without these findings [42]. The International Encephalitis Consortium has published guidelines for identification of encephalitis that might decrease this bias by using a combination approach specifically designed to include autoimmune causes [43] and some recent studies using these less strict criteria reported AE proportion as high as 26% among all encephalitis cases [4].

The etiology of AE is often unclear, but many cases has been associated with pseudo neural expression in teratomas [44] and results of several studies suggest that viral infections, and particularly HSV are implicated in its pathogenesis [45, 46]. In the current study, using quantitative RT PCR/PCR and RNA- and DNA-based metagenomics, we detected viral sequences in CSF from 3 out of 8 patients with AE. To our knowledge this is the first study employing shotgun metagenomics for the analysis of CSF from patients with AE. The role of antecedent viral infection among our AE patients could have been overestimated due to the
aforementioned inclusion criteria as higher pleocytosis and protein values are typically associated with viral etiology.

HSV-1 infection was previously associated with AE and particularly with anti-NMDAR receptor encephalitis [45, 47, 48]. A close association between HSV and AE is also suggested by the results of a retrospective study finding anti-NMDAR antibodies in 13 (30%) out of 44 patients with HSV-1 encephalitis [49]. Similarly, Salovin et al. found that markers of past HSV-1 infection are more common in anti-NMDAR encephalitis than in age-matched controls with other neuroinflammatory disorders.[50] While the exact mechanisms are still speculative, the most widely accepted hypothesis is molecular mimicry between virus-associated antigens and the NMDA receptor [17, 48]. Similar mechanism was also postulated to be behind association between HSV infection and such autoimmune diseases as stromal keratitis [51] and myasthenia gravis [52].

Importantly, the presence of anti-NMDAR was described in patients with relapsing form of HSV encephalitis in which neurological symptoms develop few weeks or even months after the initial infection and this disease is at times indistinguishable from AE [53]. Furthermore, anti-NMDAR are also found in the course of uncomplicated HSV encephalitis in as many as 30% of all patients [49] pointing to the potential for autoimmunity inherent in this infection. It is plausible that similar effects could be induced by other viral pathogens and could at least occasionally lead to full-blown AE. However, in many patients anti-NMDAR could represent nothing more than a transient epiphenomenon related to viral neuroinfection.

Another viral pathogen identified in one of our patients belonged to the Enterovirus genus. Enteroviral infection is a common cause of encephalitis and in California Encephalitis Project this particular etiology constituted 25% of all viral encephalitis cases [38]. Although association between enteroviral infection and AE was not reported previously, Nakajima et
al. described a case of a female patient with chronic progressive enteroviral limbic encephalitis who developed anti-NMDA epsilon2 receptor antibodies [54].

Metagenomic analysis detected TTV infection in one of our AE patients, and this finding was confirmed by specific PCR. TTV is highly prevalent in the general population and is considered to be an orphan virus. However, recent NGS-based studies reported on the presence of TTV in CSF of patients with encephalitis/meningitis [55-57]. Interestingly, TTV was previously associated with such human autoimmune conditions as bullous pemphigoid and lupus erythematosus [58, 59].

While metagenomics allowed for the unexpected detection of TTV it did not detect HSV sequences, which were found by our routine amplification assays. This inconsistency could be due to general lower sensitivity of metagenomic workflows compared to real time RT-PCR/PCR assays. Using serial dilutions of HIV and HSV positive sera in negative CSF, we have previously established that the limit of detection for RNA- and DNA-based metagenomics was no better than $10^2$ and $10^3$ viral copies/reaction, respectively [60].

While the presence of anti-NMDAR in CSF in association with clinical symptoms is considered to be specific in diagnostic for NMDA receptor AE [61] most of our patients did not fit the typical characteristics of AE as they were older, had low mononuclear cell CSF count and only one was serum positive. Serum negative AE represent 15% of all anti-NMDAR encephalitis cases and such patients were reported to be older, have milder neurologic symptoms and lower frequency of tumors thus being similar to our AE population [62].

In conclusion, we detected the presence of HSV, TTV and Enterovirus genus in CSF samples from AE patients. These findings support the concept of viral involvement in the pathogenesis of this disease.
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**Author contributions.** All authors have seen and approved the manuscript and contributed significantly to the work.

**Potential conflicts of interest.** All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.
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Table 1. Demographic, clinical, laboratory and NGS-based metagenomic data in patients with autoimmune encephalitis.

| Pt. | Age | Gender | AE-in CSF | Clinical manifestation | GCS | CSF cytosis/µl (lymf) (%)** | PCR in CSF | NGS workflow | Reads after trimming | Viral reads | % of viral reads | Phage/all viral species (%) | Eukaryotic viruses in NGS |
|-----|-----|--------|-----------|------------------------|-----|-----------------------------|-----------|--------------|---------------------|-------------|----------------|-----------------------------|--------------------------|
| S1  | 59  | M      | NMDAR+    | headache, fever, seizures, meningeal signs, VII nerve palsy, hearing impairment | 15  | 24 (80)                     | neg.      | DNA          | 15,234,632          | 38,360      | 0.252           | 71.67                       | neg                      |
|     |     |        |           |            |                 |           |             | RNA          | 15,369,597          | 4,316       | 0.028           | 9.52                        | neg                      |
| S2  | 22  | F      | NMDAR+    | headache, fever, seizures, psychosis, myoclonus, peripheral nerve palsy | 6   | 208 (10)                    | TTV       | DNA          | 11,359,791          | 8,728       | 0.077           | 64.71                       | TTV                      |
|     |     |        |           |            |                 |           |             | RNA          | 17,488,272          | 8,555       | 0.049           | 10.0                       | neg                      |
| S3  | 21  | M      | NMDAR+*   | headache, fever, seizures | 13  | 20 (99)                     | EV***     | DNA          |                        |             |                 |                             | No metagenomic analysis |
|     |     |        |           |            |                 |           |             | RNA          |                        |             |                 |                             |                         |
| S4  | 55  | F      | NMDAR+    | headache, memory disorders | 14  | 76 (95)                     | neg.      | DNA          | 12,276,106          | 10,959      | 0.089           | 75.72                       | neg                      |
|     |     |        |           |            |                 |           |             | RNA          | 14,894,031          | 46,880      | 0.315           | 16.67                       | neg                      |
| S5  | 62  | F      | GABA+     | seizures, memory disorders | 8   | 72 (95)                     | neg.      | DNA          | 13,558,199          | 1,805       | 0.013           | 75.92                       | neg                      |
|     |     |        |           |            |                 |           |             | RNA          | 17,233,575          | 16,954      | 0.098           | 22.73                       | neg                      |
| S6  | 20  | F      | NMDAR+    | seizures, psychosis | 13  | 23 (51)                     | neg.      | DNA          | 12,950,423          | 4,259       | 0.033           | 75.61                       | neg                      |
|     |     |        |           |            |                 |           |             | RNA          | 13,407,464          | 4,735       | 0.035           | 60.71                       | neg                      |
| S7  | 27  | F      | NMDAR+    | seizures, psychosis, memory disorders, peripheral nerve palsy | 10  | 2 (81)                      | neg.      | DNA          | 12,931,073          | 1,101       | 0.009           | 44.44                       | neg                      |
|     |     |        |           |            |                 |           |             | RNA          | 10,793,065          | 4,310       | 0.040           | 5.56                        | neg                      |
| S8  | 20  | F      | NMDAR+    | fever, seizures, psychosis, myoclonus | 10  | 2 (63)                      | HSV-1 (300c/ml) | DNA          | 12,243,743          | 132,884     | 1.085           | 72.41                       | neg                      |

nd: not done, neg: negative; GCS: Glasgow Coma Scale
*anti-NMDAR were detected in CSF and serum
** counted in one ml of CSF
***EV: assay was detecting Coxsackie A9, A16, B2, B3, B4, B5; ECHO 5, 6, 9, 11, 18, 30 and enterovirus 71