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BRIEF REPORT

A new densovirus in cerebrospinal fluid from a case of anti-NMDA-receptor encephalitis

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Abstract We characterized the genome of a densovirus, tentatively called human CSF-associated densovirus 1 (HuCSFDV1), in cerebrospinal fluid (CSF) from a human case of encephalitis with antibodies against the N-methyl D-aspartate receptor. The presence of the viral genome in CSF was independently confirmed. This virus, which is proposed to be a member of a new species in the genus *Iteradensovirus* of the subfamily *Densovirinae*, showed the typical two ORFs encoding nonstructural and structural proteins with low-level identities of 22 and 16 % to the closest known densovirus relative. No other eukaryotic viral sequences were detected using deep sequencing. The replication and pathogenicity in humans of this virus, which belongs to a viral subfamily whose members are only known to replicate in invertebrates, remain to be demonstrated. Alternative explanations for the detection of densovirus DNA in CSF are discussed.

Densoviruses are small non-enveloped viruses with linear single-stranded DNA genomes ranging from 4 to 6 kb belonging to the subfamily *Densovirinae* within the family *Parvoviridae*. Members of the *Densovirinae* are known to infect invertebrates and are responsible for several diseases in their hosts [2]. The other *Parvoviridae* subfamily is the *Parvovirinae,* whose members infect mammals and birds. These subfamilies differ most prominently in the relative orientation of their two major genes, NS1 and VP. A new densovirus was recently associated with an extensive outbreak of sea-star wasting disease on the west coast of North America [11]. Densoviruses are also frequently detected in mosquitos, showing variable degrees of pathogenicity [3, 23]. Densovirus sequences have also been reported in feces of rodents [19], bats [8, 14], and camels [30], likely because of the presence of viruses in their diets.

Viral metagenomics was used to analyze 53 CSF specimens collected from patients with encephalitis without identified infectious etiology at Children's Hospital Colorado (CHCO) in Aurora, CO, USA. This study was approved by the Colorado Multiple Institution Review Board, and informed consent was obtained from all subjects. Pools of three to five CSF specimens were clarified by centrifugation at 15,000×*g* for ten minutes and then filtered through a 0.45-μm filter (Millipore). The filtrates were treated with a mixture of nuclease enzymes to digest unprotected nucleic acids. Viral nucleic acids were extracted using a Maxwell® 16 automated extractor (Promega). cDNA and DNA were then generated by using random RT-PCR followed by the use of a Nextera XT Sample Preparation Kit (Illumina) to construct a DNA library with each pool identifiable using dual barcodes. The library was deep-sequenced using the MiSeq Illumina platform with 250 base paired ends. Viral sequences were identified through translated protein sequence similarity search (BLASTx) comparing them to those of annotated viral proteins available in GenBank's viral database.

Using a BLASTx cutoff of E scores $<10^{-5}$, one pool of five CSF samples generating 3×10^6 sequence reads

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showed 221 reads related to densovirus (GenBank SRP075630). Total nucleic acids of each individual specimen within that pool were extracted, and the individual specimen containing the densovirus DNA was identified by PCR. Primers Huca-F1 (5′-CTG GAA GCG ACG AAG ATG GAC T-3′) and Huca-R1 (5′-CTT TGT CCA TCT CCT CTG CGG TG-3′) were used for the first round of PCR, and primers Huca-F2 (5′-ACT ATG CTC TCC CTT TCC GAC-3′) and Huca-R2 (5′-CAT ACT TGT CTC CAG GAA TCT TGT C-3′) were used for the second round of PCR. The PCR conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 54 °C (for the first round) or 50 °C (for the second round) for 30 s and 72 °C for 1 min, a final extension at 72 °C for 10 min, resulting in an amplicon of 406 bp.

The patient was a 6-year-old girl who presented in February with her second of three episodes of recurrent encephalitis with altered mental status, aphasia, facial droop, and hemiplegia. The patient had not traveled, had no animal exposures, and had no known insect bites. Lumbar puncture was obtained three days after onset, and the sample contained 52 white blood cells/mm^3 (74 % neutrophils, 16 % lymphocytes, 10 % monocytes), 14 red blood cells/mm^3, normal glucose and normal protein. Magnetic resonance imaging demonstrated diffuse bilateral hyperintensity throughout the cerebral sulci on FLAIR sequences and gyral swelling within the right frontal, right occipital, and left insular lobes. An electroencephalogram demonstrated diffuse encephalopathy. Anti-N-methyl D-aspartate (NMDA)-receptor antibodies were identified by indirect fluorescent antibody and cell-based assay in CSF [5, 6]. Pelvic ultrasound was negative for ovarian tumors. The patient was treated with high-dose intravenous steroids and rituximab, which resulted in some improvement, but she had significant persistent medical problems requiring 7 weeks of inpatient rehabilitation. Of note, 20 months later, the patient had a third episode of encephalitis with seizures, MRI changes, and CSF pleocytosis but returned to baseline without treatment in 3 days.

The nearly complete genome sequence of a new densovirus was determined by *de novo* assembly of densoviral reads generated from deep sequencing, and this virus was named human CSF-associated densovirus (HuCSFDV). Putative ORFs were identified using the NCBI ORF finder. Sequences beyond the major ORFs were considered 5′ and 3′ UTRs. Alignments and phylogenetic analyses were based on the translated amino acid sequences. Sequence alignment was performed using CLUSTAL X. Sequence identities were determined using BioEdit. Phylogenetic analyses were performed using NS1 proteins of the most closely related densoviruses [4]. Phylogenetic trees with bootstrap resampling of the alignment data sets were generated using the maximum-likelihood method and visualized using the program MEGA version 6 [24]. The bootstrap value for each node is shown if >70 %.

The sequence of the HuCSFDV genome determined here (GenBank KX035107) was 4,678 bases in length, including a partial 5′ UTR (317 bases), regions encoding nonstructural proteins (NS1, NS2 and NS3) and a structural protein (VP1), and a partial 3′ UTR (120 bases). The VP1 of HuCSFDV was 840 aa in length and showed the highest identity of 16 % to that of a densovirus infecting the cotton bollworm moth (*Helicoverpa armigera*) in the genus *Iteradensovirus* [31]. The VP1 protein contained the phospholipase A_2 (PLA_2) motif found in other members of the genus *Iteradensovirus*. The putative NS1 (568 aa) resulted from a predicted spliced transcript in which a potential splice donor site CG↓GT and a potential splice acceptor site AG↓G were identified (Fig. 1A) using Alternative Splice Site Predictor [28]. The branch site (AATTGAC) was also found in the intron. Upstream (5') from the splice acceptor site AG↓G, a region high in pyrimidines (TATCATTTT) was detected. Based on sequence comparison to known iteradensoviruses (Fig. S1), two replication initiator motifs (^{137}HYHFLHH^{143} and ^{180}QLFYVHKGA^{188}) and three helicase motifs (^{399}KENTLQIVSPPSSGKNFFFDPIFLY^{423}, ^{449}ILYWNEPNFE^{458}, and ^{489}TPVIITTN^{496}) were identified in NS1 [12, 26, 27]. A phylogenetic tree based on the NS1 protein sequences of HuCSFDV and the representatives of five genera in the subfamily *Densovirinae* is shown (Fig. 1B) [4]. The NS1-based phylogenetic analysis demonstrates that HuCSFDV shared a common root, although in a basal position, with members of the genus *Iteradensovirus*. The NS1 protein showed the highest identity of 22 % to that of a densovirus infecting cotton bollworms (*Helicoverpa armigera*) [31] (Fig. 1C). Taken together, HuCSFDV (GenBank KX035107) is therefore proposed as prototype of a new species of the genus *Iteradensovirus* pending ICTV review. Members of the genus *Iteradensovirus* are known to infect members of the genus *Lepidoptera* (butterflies and moths), and related endogenized genome fragments have been detected in the chromosomes of ticks [15].

In order to confirm the presence of HuCSFDV in the CSF sample and exclude the possibility of local DNA contamination, the same PCR protocol was used for independent testing at CHCO, using a CSF aliquot retained on site, and this confirmed the presence of HuCSFDV DNA in the original CSF sample. The same PCR assay targeting NS1 was then used to determine the detection rate of HuCSFDV in all 53 CSF specimens in Colorado, USA. No other specimens except the one initially detected by deep sequencing were PCR positive, yielding a low detection rate of 1.9 % (1/53) in this population. To investigate the possibility of HuCSFDV viremia, plasma samples from this patient were also tested by nested PCR for HuCSFDV DNA. The blood specimens from both the acute and convalescent phases, which were drawn one day before and

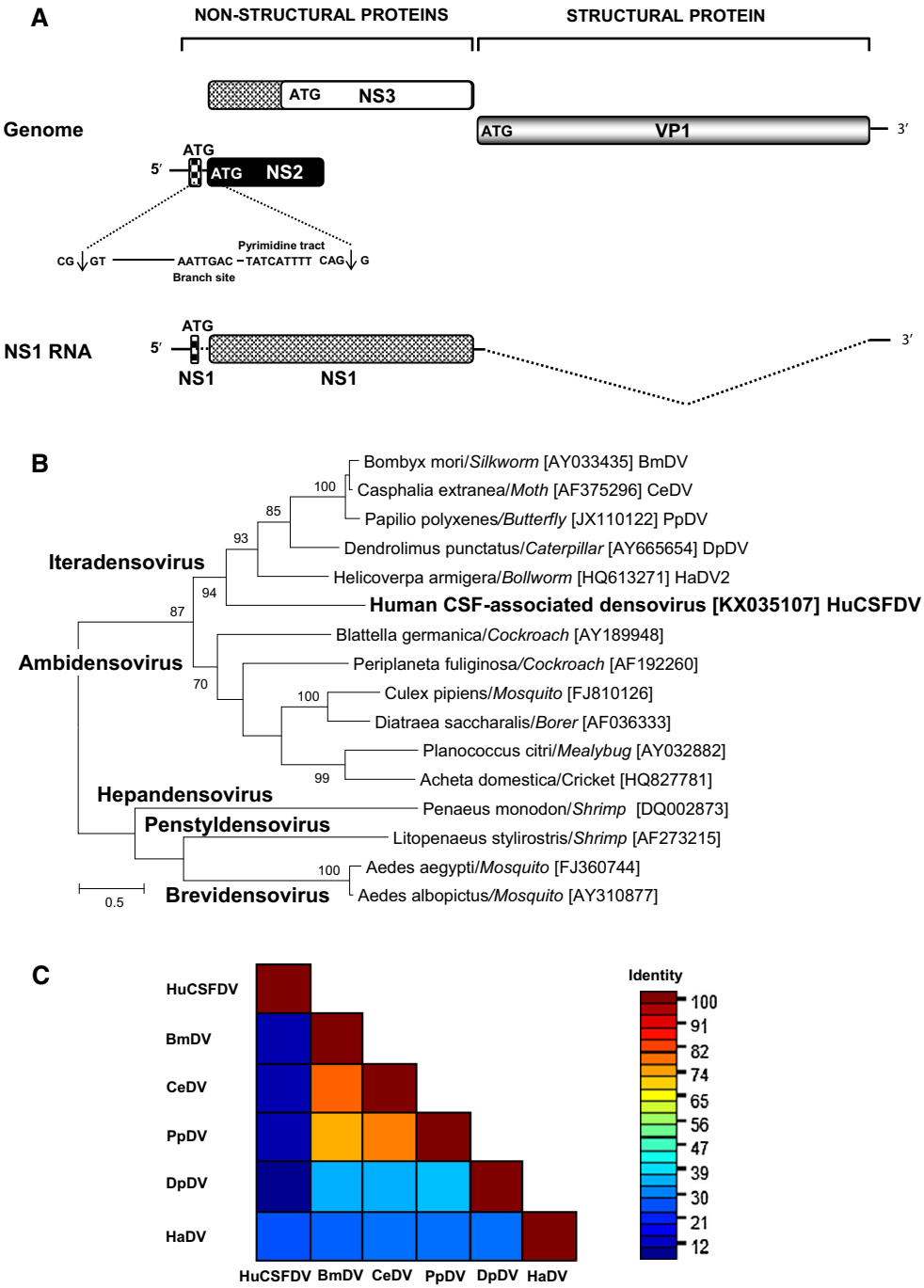

Fig. 1 A new densovirus genome and phylogeny. **(A)** Organization of the human CSF-associated densovirus genome. Theoretical splicing for expression of NS1 is shown. **(B)** A phylogenetic tree generated based on NS1 sequences of human CSF-associated densovirus and the representatives of five genera in the subfamily *Densovirinae*. The scale indicates amino acid substitutions per position. Bootstrap values (based on 100 replicates) for each node are given if >70. **(C)** Pairwise comparison of NS1 protein sequences of human CSF-associated densovirus and the representatives of five species in the genus *Iteradensovirus*

nine months and thirteen days after collection of CSF, were PCR negative for HuCSFDV DNA.

In an attempt to identify the presence of other viruses, the HuCSFDV-positive specimen was then analyzed individually (i.e., not in a pool) using the same metagenomics approach (GenBank SRP075630). The HuCSFDV genome was again sequenced, and no other eukaryotic viral sequences were detected.

It is possible to speculate about the origin of a densovirus genome in CSF. Does HuCSFDV expand the known cellular host range of densoviruses from invertebrates to include mammals and play a role in this patient's neurological symptoms? The detection of endogenized parvoviruses in numerous vertebrates as well as tunicates (sea squirt) and of endogenized densoviruses in insects as well as crustacean, arachnids, and flatworms indicates that the host range of members of the subfamilies *Parvovirinae* and *Densovirinae* may be broader than previously appreciated [15]. The absence of endogenized densovirus genes in the vertebrate genomes analyzed so far makes it

unlikely that densovirus are able to replicate in vertebrates [15].

Could HuCSFDV be a trigger for anti-NMDA receptor antibodies through molecular mimicry, similar to what has been theorized for herpes simplex virus [1, 9, 13, 25, 29]? Alignment of the viral proteins with the NMDA receptor protein did not show regions of strong similarity, particularly surrounding the N368/G369 region of the GluN1 amino-terminal domain of the epitope conferring antigenicity [10]. Could detection of HuCSFDV DNA in CSF reflect its passive transfer from an undetected insect bite [7, 17, 21, 22, 32] or human infection with an invertebrate in which this densovirus was replicating and releasing viral particles into the CSF? Alternatively, sample contamination could also conceivably occur from the skin or an environmental source during CSF sample collection. Another densovirus genome (Acheta domesticus densovirus in the genus *Ambidensovirus*) [16] was recently detected using metagenomics on nearly all tested skin surfaces of a healthy person over a 16-month period [18]. The role, if any, and the cellular origin of HuCSFDV in this case of encephalitis therefore remains unknown but further demonstrates the diversity of viral genomes detectable in clinical CSF samples using deep sequencing [20, 33].

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Compliance with ethical standards

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Conflict of interest All authors declare that there is no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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