Comprehensive Neuropathologic Analysis of Genetic Prion Disease Associated With the E196K Mutation in PRNP Reveals Phenotypic Heterogeneity

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Abstract

The genetic forms of human transmissible spongiform encephalopathies (TSEs) are linked to mutations in the gene encoding the prion protein (PRNP) and account for 10% to 15% of human TSE cases. Some are distinct with respect to clinical signs, disease onset/duration, and diagnostic findings, whereas others closely resemble sporadic Creutzfeldt-Jakob disease (sCJD). We report a comprehensive analysis of 4 patients carrying the rare E196K (GAG→AAG) mutation who presented with clinical features of CJD. To date, information on this PRNP mutation is limited to clinical and genetic data. Consequently, the E196K mutation could not be unequivocally assigned to human prion disease. We report histopathologic and biochemical findings in addition to clinical observations, thus providing a more comprehensive analysis of this presumably genetic prion disease. Our data indicate that (i) the E196K mutation is causally linked to human prion disease, (ii) there is a complex phenotypic spectrum of this mutation that includes nonspecific symptoms at onset and features typical of sCJD during disease progression, and (iii) the corresponding histologic picture comprises both cases with atypical neuropathology and cases that closely resemble subtypes of sCJD corresponding to the classification of Parchi et al, with subtle modifications in hippocampal regions CA1-4.

Key Words: Creutzfeldt-Jakob disease, E196K, Prion, PRNP, PrPSc, Transmissible spongiform encephalopathy.
We performed a comprehensive study of 4 patients carrying the PRNP mutation E196K and report for the first time the histopathologic and biochemical features associated with the E196K mutation.

MATERIALS AND METHODS

Selection of Cases

Four patients carrying PRNP mutation E196K had been identified as suspected CJD cases to the German CJD Surveillance Unit based on current clinical criteria for CJD (20–22); the diagnoses were verified at autopsy (Table). Clinical data include results of physical examination, electroencephalography (EEG), magnetic resonance imaging (MRI), and evaluation of biomarkers in the cerebrospinal fluid (CSF) such as protein 14-3-3, Tau, neuron-specific enolase (NSE), and glial protein S100b (23–26). The investigations were performed with informed consent and following all the institutional guidelines for experimental investigations with human specimens.

Mutation Analysis and Genotyping

Genomic DNA was extracted from blood samples and the coding region of PRNP was amplified by polymerase chain reaction. Subsequently, the complete open reading frame of PRNP was screened for mutations by direct sequencing using the automated LICOR 4200 DNA Analyzer/Genetic Analyzer 3130 (Applied Biosystems, Lincoln, NE), as previously described (27, 28). Briefly, the amino acid at codon 129 in coupling with the mutation was determined by cloning. The polymerase chain reaction product was cleaved with EcoRI and XmaI. Subsequently, the large fragment was purified and cloned into the appropriate cleavage sites of pBluescriptIKS(−) (Stratagene, La Jolla, CA). The mutation and the respective codon 129 were verified by sequencing of 3 independent clones.

Histopathologic and Immunohistochemical Analyses

Autopsy and sampling of material for histopathologic analyses of various brain regions were performed as previously described (1). In addition, frozen samples of the frontal cortex, hippocampus, and cerebellum were preserved for biochemical analysis. Control brain tissue was obtained from corresponding brain regions of sCJD patients with the MM 1, VV 2, and MV 2 subtypes. Brain material for histopathologic analysis was fixed in 4% buffered formalin, inactivated with 100% formic acid for 1 hour, fixed in formalin

<table>
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<th>TABLE. Clinical Features of E196K Patients</th>
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<td>CJD typical EEG (periodic sharp waves)</td>
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<td>Other symptoms</td>
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<td>CJD typical MRI (hyperintense basal ganglia or cortex)</td>
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+, present; −, not conspicuous/absent; CJD, Creutzfeldt-Jakob disease; EEG, electroencephalogram; M, methionine; MRI, magnetic resonance imaging; NA, not available; NSE, neuron-specific enolase; V, valine.

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were subjected to sodium dodecylsulfate (CDP-Star; Roche, Mannheim, Germany). PrPSc glycoform was detected, using PrP-specific antibody L42 (30). For the detection of hyperphosphorylated Tau protein, specific antibody AT8 (1:200; Perbio, Frankfurt, Germany) was used. Detection of α-synuclein was performed using antibody 15G7 (1:600; Novoceastra, Newcastle upon Tyne, UK). Histologic sections were evaluated blinded and scored semiquantitatively for spongiosis, gliosis, and neuronal loss independently by 2 neuropathologists. PrPSc levels in hippocampal regions were compared for spongiosis, gliosis, and neuronal loss independently.

Immunoblot Analyses

Tissues from the frontal cortex, hippocampus, and the cerebellum were homogenized in 9 volumes (wt/vol) of lysis buffer containing 100 mmol/L sodium chloride, 10 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium deoxycholate, 0.5% octylphenoxypolyethoxyethanol (Nonidet P-40), 100 mmol/L tris(hydroxymethyl)aminomethane, pH 6.9, at 37°C. After PK digestion (100 μg/mL, 1 hour at 37°C), homogenates were subjected to Sodium dodecylsulfate–polyacrylamide gel electrophoresis using precast 12% Bis-Tris gels (NuPAGE; Invitrogen, Karlsruhe, Germany). For classification and comparison of PrPSc types, we used brain homogenates from sCJD patients with PrPSc types 1 (129 MM) and 2 (129 VV and 129 MV), respectively. The monoclonal antibody 3F4 (1: 3.000; DAKO, Hamburg, Germany), recognizing the epitope 109–112 of human PrP, was used for immunodetection. Immunoreactivity was visualized by enhanced chemiluminescence (CDP-Star; Roche, Mannheim, Germany). PrPSc glycoform ratios were evaluated densitometrically using AIDA image analyzer (Raytest, Straubenhardt, Germany).

RESULTS

Clinical

Patient 1

A 77-year-old woman from the North of Germany was admitted to hospital with the working diagnosis of brainstem infarction/internal hydrocephalus by her general practitioner. At that point, the patient had a 6-week history of gait instability, confusion, and impaired short-term memory. She also had a history of diabetes mellitus accompanied by peripheral polyneuropathy. In the following 2 weeks, she showed progressive dementia and finally akinetic mutism. The EEG revealed severe grouped dysrhythmia, but periodic sharp-wave complexes typical of CJD were absent. Examination of her CSF revealed slightly elevated NSE (30.5 μg/L). There was no record of other measurements of other CSF biomarkers associated with CJD. The patient was classified as “possible CJD” according to clinical criteria (20–22). The family history was negative for dementia or a neurodegenerative disease. The patient’s mother had died at the age of 83 with pneumonia, and her father had died at the age of 70 after a stroke. The patient died after a disease duration of 2 months. Direct sequencing of the PRNP gene open reading frame revealed the E196K mutation and homozygosity for methionine at codon 129.

Patient 2

A 72-year-old man from Southern Germany was admitted to the hospital for etiologic clarification of dementia. The past medical history revealed chronic alcohol abuse. Initially, the patient was diagnosed with Wernicke encephalopathy. Two months later, his wife reported worsening of symptoms that included visual hallucinations, speech, and walking difficulties and incontinence. On physical examination, the patient was drowsy but awake. Neurologic examination demonstrated ataxia, rigor, dysarthria/anarthria, and myoclonus. Electroencephalography revealed typical periodic sharp-wave complexes. Cerebrospinal fluid proteins 14-3-3 were detected, but there were no other relevant CSF protein measurements recorded. Magnetic resonance imaging showed mild cerebellar and parietal atrophy but no typical signs of CJD. The patient was classified as “probable CJD” according to clinical data. He developed akinetic mutism and died after a disease duration of 2.5 months. Direct sequencing of the PRNP gene open reading frame revealed the E196K and homozygosity for valine at codon 129. The family history was negative for dementia or a neurodegenerative disease.

Patient 3

A 74-year-old woman from Southwest Germany first developed severe bifrontal headache paralleled by depression and loss of appetite. In the following months, she developed ataxia associated with dysarthria and dysphagia and, later, myoclonus and akinetic mutism. Because of her markedly impaired communication ability, dementia could not be assessed. The EEG indicated the appearance of periodic sharp-wave complexes, and CSF levels of 14-3-3, Tau (19,537 pg/mL), NSE (113.8 ng/mL), and S100b (16.2 ng/mL) were elevated. Magnetic resonance imaging showed white matter lesions termed multilocular leukoencephalopathy and a general atrophy but no typical features of CJD. On the basis of clinical data, “probable CJD” was diagnosed before the genetic analysis was available. Direct sequencing of PRNP revealed heterozygosity for methionine and valine (MV) at codon 129. Subsequent cloning experiments demonstrated coupling of the E196K mutation with methionine at codon 129. The patient died after a disease duration of 4 months. There was no family history of a neurologic or psychiatric disorder.
Patient 4

A 69-year-old woman from Eastern Germany had a 6-month history of tremor accompanied by signs of dementia for 3 weeks before she was hospitalized. On admission, she additionally showed rigor, bradykinesia, cogwheel phenomenon, and small step gait and was first diagnosed with Parkinson disease. The patient reported a lack of concentration and considerable weight loss without reduced appetite in the last months. The EEG showed triphasic waves predominantly on the right. Magnetic resonance imaging revealed signal hyperintensities in the frontoparieto-occipital cortex and in the basal ganglia, and white matter lesions and general atrophy were also observed. In the CSF, the proteins 14-3-3 were detectable, and Tau was elevated (>1200 pg/mL); S100 protein level was unremarkable. In the course of disease, she developed ataxia, dysarthria, and apraxia and finally showed the picture of akinetic mutism, with pyramidal and extrapyramidal signs as well as myoclonus. The clinical diagnosis was “probable CJD.” The patient died after a disease duration of 8 months. Direct sequencing of the PRNP gene open reading frame revealed the E196K mutation and homozygosity for methionine at codon 129. Importantly, there was a positive family history: the patient’s brother was diagnosed with probable genetic CJD (E196K mutation) and died at age 50 years. Detailed clinical data are not available for this patient, and no autopsy was performed. Nevertheless, these data suggest autosomal dominant inheritance of this putative pathologic mutation.

Neuropathologic and Immunohistochemical Findings

Histologic examination of all 4 cases demonstrated morphologic and immunohistochemical features of CJD. The findings in Patients 1 (129 MM) and 3 (129 MV) resembled those of the sCJD MM/MV 1 type with mild to severe spongiform degeneration, astrocytic gliosis, and focal loss of neurons, mainly affecting the cerebral cortex, basal ganglia, thalamus, and cerebellum (Fig. 1, left column); the brainstem was unaffected. In contrast, the hippocampal regions CA1-4, which are usually spared in the MM/MV 1 type (1), were also affected by mild spongiform changes (Fig. 1, left column). Immunohistochemistry revealed a synaptic pattern of PrPSc deposition in the affected areas including regions CA1-4 of the hippocampus (Fig. 1, left). The latter was even more obvious on PET blot sections (Fig. 2A). Patient 3 additionally showed a distinct microvasculopathy in the white matter, but the neuropathologic findings were similar to those in Patient 1 (not shown).

Patient 2 (129 VV) had mild to moderate spongiform degeneration, astrocytic gliosis, and neuronal loss with laminar distribution in the deep cortical layers as well as prominent involvement of basal ganglia, thalamus, cerebellum, and brainstem nuclei (Fig. 1, middle column). The hippocampus, which is characteristically involved in the VV 2 subtype (1), was severely affected (Fig. 1, middle column). Immunostaining revealed synaptic and perineuronal PrPSc deposits in the affected areas, predominantly in the deep cortical layers and the hippocampus. The cerebellum showed intense plaque-like PrPSc deposits not only in the molecular and granular cell layers but also in the white matter (Fig. 1, middle column). Involvement of the hippocampus was also demonstrated on PET blot sections (Fig. 2A).

Patient 4 (129 MM) showed a different pattern: spongiform changes, gliosis, and neuronal loss were more severe but in general followed the same morphology and distribution as in Patients 1 and 3 (Fig. 1, right column). There was also mild microvasculopathy in the white matter. Immunohistochemistry displayed clear synaptic PrPSc staining in the affected areas including regions CA1-4 of the hippocampus (Fig. 1, right). The latter was even more obvious on PET blot sections (Fig. 1). Patient 3 additionally showed a distinct microvasculopathy in the white matter, but the neuropathologic findings were similar to those in Patient 1 (not shown).

FIGURE 2. Paraffin-embedded tissue (PET) blot analysis and quantification of PrPSc deposits in the hippocampus of E196K patients and corresponding sporadic Creutzfeldt-Jakob disease (sCJD) controls. (A) PrPSc deposits in the hippocampus including regions CA1-4 compared with sCJD subtype MM 1 and VV 2 controls. The black line in the sCJD MM 1 control indicates the typical “border” between strong PrPSc deposits in the subiculum (S) in contrast to the almost spared regions CA1-4. (B) Densitometric quantification of PrPSc deposits in regions CA1-4 of MM/MV subjects normalized to the intensity of the subiculum (S). (C) Corresponding quantification of PrPSc deposits in regions CA1-4 of VV subjects. Black bars indicate E196K cases; empty bars, sCJD controls.
129 VV patients, the CA1-2 region is also more affected in sCJD (1).

All cases were additionally examined for possible coexistent synucleinopathies and/or tauopathies. Immunohistochemistry using an anti-synuclein antibody was negative for pathologic deposits in all patients, No hyperphosphorylated tau-positive structures other than very few neurofibrillary tangles in the transentorial region of Patients 2 and 4 (corresponding to Braak stage I [31]) were detected (not shown).

**Biochemical Analysis of the E196K Mutation in PRNP**

The presence of PrPSc was demonstrated by Western blot analysis in 3 different brain regions (frontal cortex, cerebellum, and hippocampus) in the 4 patients (Figs. 3 and 4). According to the Western blot classification of PrPSc types by Parchi et al (11, 12), the PK-resistant PrP (PrP27-30) found in Patients 1 (129 MM) and 3 (129 MV) was PrPSc type 1 (Figs. 3A, C), whereas Patient 2 (129 VV) presented with PrPSc type 2 (Fig. 3B). For Patient 1, Western blot analysis in the cerebellum consistently resulted in poor band quality, which we attributed to tissue preservation because this case the cerebellum consistently resulted in poor band quality, which we attributed to tissue preservation because this case was otherwise indistinguishable from Case 3. Notably, Western blot analysis of the neuropathologically “atypical” Patient 4 (129 MM) revealed PrPSc type 2 in all samples and, in addition, PrPSc type 1 in the frontal cortex (Fig. 3D, lane 4) and the occipital lobe (not shown). Importantly, this patient does not resemble the sCJD cases with so-called “mixed phenotype” and coexistence of PrPSc types 1 and 2 (13), which typically have PrPSc type 1 in the cerebellum and perivascular deposits associated with confluent vacuoles in the cortex, whereas this patient had PrPSc type 2 in the cerebellum and 129 VV patients, the CA1-2 region is also more affected in sCJD (1).

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**FIGURE 3.** Western blot analysis of proteinase K (PK)-treated homogenates extracted from 3 different brain regions from the 4 affected E196K subjects. (A–D) Frontal cortex homogenates from sporadic Creutzfeldt-Jakob disease (sCJD) subtypes classified as MM 1 (lane 1), VV 2 (lane 2), and MV 2 (lane 3) were used as controls. PK-resistant PrP from Patient 1 (A), Patient 2 (B), Patient 3 (C), and Patient 4 (D) Positions of the molecular mass markers are indicated (kDa). Cb indicates cerebellum; Fx, frontal cortex; Hip, hippocampus.

**FIGURE 4.** Relative abundance of the 3 glycoforms of proteinase K (PK)-resistant PrP in the 4 patients carrying the E196K mutation versus sporadic Creutzfeldt-Jakob disease (sCJD) controls (Co). The relative abundance of the diglycosylated (upper) band is depicted in white, the monoglycosylated (middle) band is shown in gray, and the nonglycosylated (lower) band is shown in black. Proportions of nonglycosylated bands were analyzed using Student t test. ***, p < 0.001; *, p < 0.05.

(Fig. 3D) and plaquelike deposits in the cortex (Fig. 5). Densitometric analysis of the PK-resistant bands showed glycoform ratios different from those found in sCJD cases with underrepresentation of nonglycosylated forms in all brain regions in all 4 E196K patients irrespective of the PrPSc type and neuropathologic phenotype (Figs. 3 and 4). Statistical analysis revealed a significant underrepresentation of nonglycosylated forms in all brain regions in all 4 patients compared with the respective sCJD controls (Patients 1, 2, and 3: p < 0.001, Patient 4: p < 0.05).

**DISCUSSION**

Little information has been published regarding the E196K mutation, and in previous reports, only clinical data were available (15–18). Thus, a definite diagnosis of CJD, which requires autopsied brain material, was not feasible. The cases reported previously were thus classified as “probable CJD” according to available clinical data. Consequently, the E196K mutation could not be unequivocally assigned to human prion disease. According to the Diagnostic Criteria Guidance from the Advisory Committee on Dangerous Pathogens’ TSE Working Group (http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@ab/documents/digitalassets/dh_087508.pdf), “Definite genetic TSE requires a neuropathological confirmation of TSE, plus either definite TSE in a first degree relative (i.e., a parent, child or sibling), or a pathogenic prion protein gene (PRNP) mutation.” We are the first to provide a neuropathologic confirmation of TSE in patients with the E196K mutation (based on histopathologic, immunohistochemical, and biochemical results); thus, our data confirm that E196K results in genetic prion disease.

Generally, the clinical picture in our 4 patients was initially characterized by nonspecific symptoms including behavioral abnormalities at disease onset that led to initial diagnoses of Parkinson disease, Wernicke encephalopathy,
and brainstem infarction/internal hydrocephalus. This non-specific initial picture is consistent with the previous reports, but nonspecific symptoms at onset are common in sCJD and sometimes lead to alternative clinical diagnoses. Although the number of cases we analyzed is small, frequent occurrence of alternative initial clinical diagnoses seems unusual to us; as the disease progressed, the patients developed typical features of CJD. Information on additional patients without neuropathologic investigations is provided elsewhere (Schelzke et al, unpublished data).

Immunohistochemical analysis ruled out an underlying α-synucleinopathy that could potentially influence the initial clinical picture, which is of particular interest with respect to Patient 4 who showed symptoms suggestive of Parkinson disease. Similarly, there was only minor tau pathology in Patients 2 and 4, making it unlikely that there was a contribution of these changes to the clinical manifestations in these patients. These observations suggest that the nonspecific clinical symptoms at the disease onset were not secondary to other neurodegenerative disease processes. However, Patient 3 had signs suggestive of microvasculopathy, and Patient 4 additionally had white matter lesions on MRI; therefore, we cannot exclude the possibility that the initial clinical syndrome, especially the parkinsonism in Patient 4 (32), was at least partially related to vascular changes in these patients.

FIGURE 5. Immunohistochemistry for pathological PrP deposition in Case 4. (A–C) PrP deposits in the frontal cortex. A predominantly synaptic deposition pattern in the gray matter can be seen with focal plaquelike deposits. Panels (B) and (C) show larger magnifications of the areas indicated in (A) with a lined box (B) and dashed box (C), respectively. (D–F) Focal synaptic and small plaquelike deposits in the cerebellar cortex. Scalebar: (A, D) = 200 μm; scalebar: (B–C, F) = 20 μm, scalebar: (E) = 50 μm.
The median onset of disease in the affected E196K subjects was 72 ± 3.4 years (mean ± SD), which is approximately 10 years later than reported for other point mutations in the PRNP gene, including the most common mutation E200K (19). This finding is consistent with previous reports (15-18). The mean duration of disease was 4 ± 2.7 months, which is shorter than that reported for other genetic TSEs ranging from 5 to 40 months (19). Thus, the affected E196K subjects had a later disease onset along with shorter disease duration.

The extensive assessment of genetic and neuropathologic findings in combination with the biochemically determined PrPSc type in sCJD patients has led to a comprehensive classification that includes 6 sCJD PrPSc subtypes (1) and also accounts for mixed phenotypes in an updated classification (13). According to this widely accepted classification of sCJD cases, we found that Patients 1 and 3 were closely related with the sCJD MM/MM 1, Patient 2 was closely related with the sCJD VV 2 subtype, whereas Patient 4 had an atypical phenotype. Densitometric analysis of the PK-resistant bands revealed a significant underrepresentation of the nonglycosylated form in all brain regions in all 4 patients carrying the E196K mutation, irrespective of the PrPSc type and neuropsychologic phenotype. Such a marked underrepresentation of the nonglycosylated form relative to the diglycosylated and monoglycosylated form is not pathognomonic for the E196K mutation as it has previously been observed in subjects with a D178N mutation coupled with methionine codon at position 129 in PRNP (33).

More precisely, the classic sCJD VV 2 subtype usually presents with spongiform changes and plaquelike/perineuronal PrPSc deposits predominantly in the deep cortical layers, hippocampal region CA4, and the cerebellum, whereas the sCJD MM/MM 1 subtype usually shows spongiform changes throughout all cortical layers as well as synaptic PrPSc staining. Patients 1, 2, and 3 closely resemble the picture typically seen in the sCJD subtypes of the corresponding codon 129 genotype (1), indicating that the phenotype associated with the E196K mutation in these 3 patients is determined by the polymorphic codon 129. Codon 129 polymorphism can influence the disease phenotype as best described for the D178N mutation coupling with methionine and as genetic CJ when coupled with valine at codon 129 in PRNP (7). In addition to this overall similarity to sCJD, the present study indicates that the E196K mutation severely affects the hippocampal CA1-4 regions.

In summary, our findings indicate that the histopathology associated with this rare PRNP mutation comprises both cases with atypical neuropathology and cases that closely resemble subtypes of sCJD corresponding to the classification of Parchi et al (1) with subtle modifications in the hippocampal regions CA1-4.

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