CHAPTER 7

EPISODIC ATAXIA ASSOCIATED WITH EAAT1 MUTATION C186S AFFECTING GLUTAMATE REUPTAKE

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ABSTRACT

Background: Episodic ataxia (EA) is variably associated with additional neurologic symptoms. At least 4 genes have been implicated. Recently, a mutation in the *SLC1A3* gene encoding the glutamate transporter EAAT1 was identified in a patient with severe episodic and progressive ataxia, seizures, alternating hemiplegia, and migraine headache. The mutant EAAT1 showed severely reduced uptake of glutamate. The syndrome was designated EA6 and shares overlapping clinical features with EA2, which is caused by mutations in *CACNA1A*.

Objective: To test the role of the *SLC1A3* gene in EA.

Design: Genetic and functional studies. We analyzed the coding region of the *SLC1A3* gene by direct sequencing.

Patients: DNA samples from 20 patients with EA (with or without interictal nystagmus) negative for *CACNA1A* mutations were analyzed.

Main Outcome Measures: We identified 1 novel EAAT1 mutation in a family with EA and studied the functional consequences of this mutation using glutamate uptake assay.

Results: We identified a missense C186S mutation that segregated with EA in 3 family members. The mutant EAAT1 showed a modest but significant reduction of glutamate uptake.

Conclusions: We broadened the clinical spectrum associated with *SLC1A3* mutations to include milder manifestations of EA without seizures or alternating hemiplegia. The severity of EA6 symptoms appears to be correlated with the extent of glutamate transporter dysfunction.

INTRODUCTION

Episodic ataxias (EAS) are rare genetic disorders characterized by recurrent episodes of cerebellar ataxia variably associated with additional neurologic features. Different subtypes of EA are defined on the basis of genetic loci and clinical manifestations.[1]

The most common and best characterized subtypes of EA are EA1 and EA2. EA1 is caused by missense mutations in the *KCNA1* gene encoding a subunit of neuronal $K_v1.1$ K^+ channels.[2] EA1 usually presents with short-lasting attacks that often are triggered by exertion, stress, or startle. Patients show persistent interictal motor unit activity (myokymia). EA2 is caused by mutations in the *CACNA1A* gene encoding the pore-forming subunit of neuronal $Ca_v2.1$ Ca^{2+} channels.[3] Mostly, nonsense, frameshift, splice site, and missense mutations have been described, resulting in either a complete loss[4] or partial impairment[5,6] of $Ca_v2.1$ channel function. The episodes in EA2 last longer than in EA1, up to several hours,[7] and are often associated with vertigo and migrainous headache and can be triggered by exercise, fatigue, and stress.[8] Acetazolamide may prevent attacks.[9] Between attacks, nystagmus usually occurs. Many patients have interictal ataxia in addition to the attacks. The EA3, EA4, and EA5 subtypes are rarer and less well-defined disorders compared with EA1 and EA2.[1]

The EA6 subtype was identified in a 10-year-old patient with a severe phenotype of episodic and progressive ataxia, seizures, alternating hemiplegia, and migraine headache.[10] A heterozygous de novo P290R missense mutation was identified in the *SLC1A3* gene by use of a candidate gene approach. *SLC1A3* encodes the glial excitatory amino acid transporter EAAT1, which is involved in glutamate removal from the synaptic cleft.[11,12] Functional analysis of the mutant EAAT1 protein showed marked reduction of glutamate uptake in vitro.[10]

In the present study, we performed a mutation analysis of the *SLC1A3* gene (OM/M 600111) in 20 patients with EA2-like symptoms but without *CACNA1A* mutations. In 1 family, we found an EAAT1 mutation that segregated with the disease in 3 patients. Functional studies revealed a moderate impairment of glutamate reuptake.

METHODS

Patients

We investigated 20 patients who were referred for molecular confirmation of EA2 in whom no mutations were found in the *CACNA1A* gene. These patients showed typical EA2-like symptoms, including interictal nystagmus but no myokymia, attacks of mild ataxia with a duration of several hours, and a positive response to acetazolamide. Except for 2 patients from the United States, all patients came from Europe, mostly the Netherlands. Family members of the proband with the *SLC1A3* mutation (Fig. 1) underwent neurologic examination by experienced neurologists (S.L.M.B. and A.H.S). All patients gave informed consent, and the study was approved by the local review board.

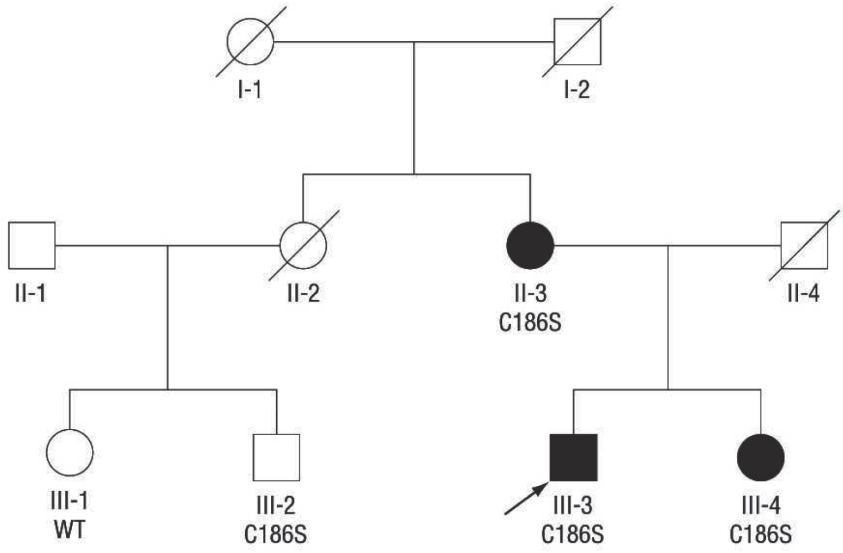

Figure 1. Pedigree of the episodic ataxia family with the EAAT1 186S mutation. Episodic ataxia is indicated by a filled black square or circle for males or females, respectively. C186S indicates heterozygosity for the mutation. WT indicates homozygosity for the wild-type allele.

Genetic studies

Genomic DNA was isolated from peripheral leukocytes using a standard salting out extraction method.[13] All exons and flanking intronic regions of the *SLC1A3* gene were amplified by polymerase chain reaction (PCR), using genomic DNA as a template. Direct sequencing was performed by cycle sequencing (Prism Big Dye Terminators Cycle Sequencing kit; Applied Biosystems, Foster City, California) using the dideoxy termination method and an ABI3700 automated sequencer (Applied Biosystems). Two hundred healthy controls were screened for the mutation by PCR analysis of exon 5 and subsequent restriction digestion of PCR products with restriction enzyme *Alu*I.

Functional studies

Functional studies[10] on glutamate uptake of wild-type and mutant EAAT1 were performed as described previously. In brief, full-length wild-type complementary DNA (EAAT1-WT) was cloned into a mammalian expression vector pcDNA3.1 (Invitrogen; Carlsbad, California). The mutant construct (EAAT1 186S) was generated by performing sitedirected mutagenesis (QuikChange; Stratagene; La Jolla, California). For functional analyses of the *SLC1A3* C186S mutation, 2 µg of wild-type (EAAT1-WT) or mutant (EAAT1-186S) EAAT1 complementary DNA constructs were transfected into COS7 cells. One day after transfection, the cells were dissociated and plated onto 60-mm-diameter tissue culture dishes. The cells were incubated with 1.5 mL of 1µM L-glutamic acid containing 1 µCi/mL of L-[3,4-3H]-glutamic acid for 2 minutes at room temperature. A total of 4 independent and masked experiments were performed, each in triplicate.

RESULTS

Genetic studies and clinical features associated with EAAT1 mutation

Mutation analysis of the *SLC1A3* gene in 20 patients revealed in 1 patient a heterozygous c.556 T_A substitution (*SLC1A3* reference sequence; GenBankNM004172) that changed a cysteine to a serine at position 186 (C186S) of the EAAT1 protein (Fig. 2A and 2B). The mutation was absent in 200 Dutch control individuals. C186S was identified in the proband (III-3), clinically affected family members II-3 and III-4, and 1 asymptomatic family member (III-2) (Fig. 1).

Clinical information of the affected family members is summarized in the Table. The proband (III-3) is a 35-year-old man who has had episodes of ataxia since early childhood. Attacks gradually changed over time. Initially, vertigo, nausea, and vomiting were the most bothersome symptoms. Later in life, truncal and gait ataxia during the attacks became more prominent. Attacks are often associated with nausea, vomiting, photophobia, phonophobia, vertigo, diplopia, slurred speech, and blurred vision. No headache was reported. Typically,

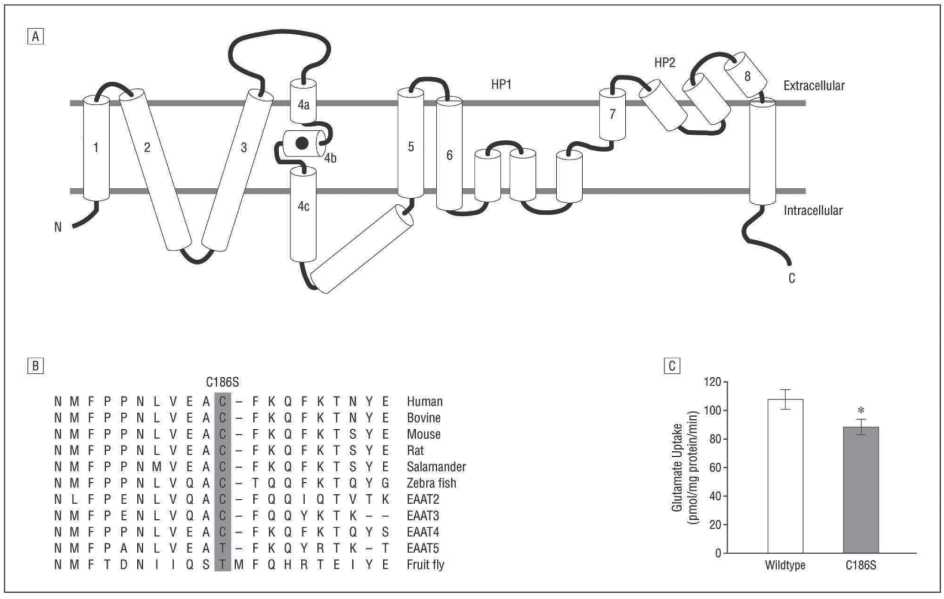

Figure 2. EAAT1 C186S mutation. (**A**) Schematic representation of the EAAT1 protein and the location of the mutated Cys186 amino acid in transmembrane segment 4b (indicated by a black dot) (the structure is adapted from Yernool et al[14]). (**B**) Conservation of the mutated residue Cys186 highlighted in gray. The protein sequences were obtained from GenBank (*Homo sapiens*, NP_004163; *Bos taurus*, NP_46411; *Mus musculus*, NP_683740; *Rattus norvegicus*, NP_062098; salamander, O57321; *Danio rerio*, NP_997805; *Drosophila melanogaster*, NP_477428; human EAAT2, AY066021; human EAAT3, NP_004161; human EAAT4, NM_005062; human EAAT5, NP_006662). (**C**) Glutamate uptake assay in COS7 cells expressing mutant EAAT1-186S (mean [SEM], 88.2[5.5]) or wild-type EAAT1-186C (mean [SEM], 107.8 [6.9]). The results are the mean (SEM) of the 4 experiments, each in triplicate. The values are picomoles of glutamate transported per milligram of protein per minute of incubation. Asterisk indicates significant reduction of glutamate uptake compared with wild type (p=0.029).
Error bars indicate SEM. HP indicates helical hairpin.

Table. Summary of clinical features of patients with episodic ataxia carrying the EAAT1 C186S Mutation

Clinical Feature	Mother (II-3)	Proband (III-3)	Sister (III-4)
Age at examination, y	56	35	28
Age of onset, y	<10	3	14
Ataxia	+	+	+
Vertigo	+	+	+
Diplopia/Visual blurring	-/-	+/+	-/-
Nausea/Vomiting	+/+	+/+	+/+
Photophobia/Phonophobia	+/+	+/+	+/-
Attack duration	Hours	Hours	Hours
Attack frequency	~ 10 / year	1-2 / month	~ 6 / year
Triggers	Emotional stress	Emotional stress, fatique, alcohol, caffeine	Emotional stress, fatique, exercise
Response to acetazolamide	+	+	+
Interictal gaze evoked nystagmus	-	+	-
Headache	-	-	+

attacks were provoked by emotional stress, fatigue, or consuming alcohol or caffeine. Attack duration was usually between 2 and 3 hours. Currently, his average attack frequency is once a month. Interictal neurologic examination revealed a horizontal gaze-evoked nystagmus without gait or truncal ataxia. Interictal electroencephalographic recording revealed no epileptic activity, and magnetic resonance imaging revealed no abnormalities (data not shown). His mother (II-3) and sister (III-4) were also diagnosed as having EAs. The 56-year-old mother (II-3) has had episodes of ataxia similar to those of the proband since elementary school. Her attacks are also associated with vertigo, nausea, vomiting, photophobia, phonophobia, and slurred speech. The attacks were not associated with headache. She now has approximately 10 attacks per year, which may last for several hours and can be triggered by stress. The 28-year-old sister (III-4) has had episodes of ataxia since the age of 14 years. Associated symptoms include vertigo, nausea, vomiting, and mild photophobia. Sometimes, the day after an attack, she experiences bilateral headache not associated with nausea, vomiting, phonophobia, or photophobia. Reported triggers are exercise, fatigue, and stress. Currently, she has on average 6 attacks a year. Typically, attacks last several hours. Acetazolamide significantly reduced the frequency of attacks in all 3 affected family members. His 40-year-old cousin (III-2) is an asymptomatic carrier of the C186S EAAT1 mutation. He experienced 4 attacks of migraine without aura and has tensiontype headache, but does not exhibit signs or symptoms related to ataxia. Individuals I-1, I-2, and II-2 were considered healthy based on limited heteroanamnestic information. His grandfather had died at the age of 98 years. His grandmother had complained about dizziness, but no neurologic examination was performed during her lifetime. No relevant clinical information is available for individual II-2, who died of an unrelated cause. Non-mutation carrier III-1 is asymptomatic.

Functional study of EAAT1 mutation C186S

To investigate the functional consequences of the EAAT1 C186S mutation, radioactive glutamate uptake assays were performed in COS7 cells. The low level of endogenous glutamate uptake activity has long established the COS7 cells as being well suited for functional studies of glutamate transporters.[15] We measured glutamate uptake in COS7 cells transfected with the wild-type (EAAT1-186C) or the mutant construct (EAAT1-186S). An 18% reduction in glutamate uptake was observed in cells expressing the mutant (mean [SEM], 88.2[5.5]) compared with the wild-type (mean [SEM], 107.8[6.9]) EAAT1, measured in picomoles per milligram of total protein per minute of incubation (p=0.029; Fig. 2C).

COMMENTS

We scanned the *SLC1A3* gene for mutations in 20 patients with EA2-like symptoms without *CACNA1A* mutations because of overlapping clinical features between EA2 and EA6. We found a novel nucleotide change c.556T_A in the *SLC1A3* gene, resulting in EAAT1 mutation C186S, in a family with EA and interictal nystagmus but without migraine, seizures, cerebellar atrophy, or alternating hemiplegia. Our genetic and functional data suggest that mutation C186S is pathogenic. First, the mutation C186S segregated with all 3 symptomatic family members but was not identified in a large panel of controls. The asymptomatic mutation carrier (III-2) had migraine without aura, but given the relatively high prevalence of migraine it is unlikely that these attacks are caused by the EAAT1 mutation. Therefore, he likely represents a nonpenetrant case of EA. Second, Cys186 is highly conserved among species (Fig. 2B). Our functional studies revealed a reduced glutamate reuptake for the mutant EAAT1 (Fig. 2C). Cys186 resides in transmembrane segment 4B (Fig. 2A) on the outer perimeter of the human EAAT1 transporter protein that is implicated in intersubunit contact.[15] The 4B-4C loop was recently shown to undergo substrate-dependent conformational changes and has been hypothesized to be important in stabilizing the trimeric structure of the transporter and coordinating the cooperativity for sodium binding.[16] Clinical severity of EA6 appears to be well correlated with glutamate reuptake capability of mutant EAAT1. The P290R mutation leads to a complete loss of glutamate reuptake and is associated with a severe EA phenotype with months-long attacks, seizures, and alternating hemiplegia.[10] In contrast, the C186S mutation has a mild effect on glutamate reuptake and is correlated with a milder EA phenotype. Although it is hard to predict from cellular studies how a mild increase in extracellular glutamate will affect cerebellar functioning in patients, it is well known that ion and neurotransmitter pathways are complex and tightly regulated. Subtle changes in these pathways have been associated with clinical manifestations.[17,18] Since we found a mutation in only 1 of 20 patients with *CACNA1A*-negative EA2-like symptoms, other genes must be involved. Likely candidate genes are components of ion and neurotransmitter pathways involved in the regulation of cerebellar neuronal excitability.

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FINANCIAL DISCLOSURE

Non reported.

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