Clinical, Neuropathologic, and Biochemical Profile of the Amyloid Precursor Protein I716F Mutation

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Abstract
We report the clinical, pathologic, and biochemical characteristics of the recently described amyloid precursor protein (APP) I716F mutation. We present the clinical findings of individuals carrying the APP I716F mutation and the neuropathologic examination of the proband. The mutation was found in a patient with Alzheimer disease with onset at the age of 31 years and death at age 36 years and who had a positive family history of early-onset Alzheimer disease. Neuropathologic examination showed abundant diffuse amyloid plaques mainly composed of amyloid-β42 and widespread neurofibrillary pathology. Lewy bodies were found in the amygdala. Chinese hamster ovary cells transfected with this mutation showed a marked increase in the amyloid-β42/40 ratio and APP C-terminal fragments and a decrease in APP intracellular domain production, suggesting reduced APP proteolysis by γ-secretase. Taken together, these findings indicate that the APP I716F mutation is associated with the youngest age of onset for this locus and strengthen the inverse association between amyloid-β42/40 ratio and age of onset. The mutation leads to a protein that is poorly processed by γ-secretase. This loss of function may be an additional mechanism by which some mutations around the γ-secretase cleavage site lead to familial Alzheimer disease.

Key Words: α-Synuclein, γ-Secretase, Alzheimer disease, Amyloid, APP mutations, Genetics.

INTRODUCTION
The genes of amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) have been implicated in the pathogenesis of familial Alzheimer disease (FAD). Mutations in the PSEN1 gene represent the most common cause of FAD (1), and more than 175 mutations have been identified to date (www.molgen.ua.ac.be/ADMutations). Mutations in the APP gene are rare but provide insight into the pathogenesis of FAD. All APP pathogenic mutations are located either in the amyloid-β (Aβ) sequence or in the vicinity of a protease cleavage site that influences APP proteolysis by different mechanisms. The Swedish APP mutation, adjacent to the β-cleavage site, increases the production of total Aβ by enhancing APP cleavage by β-secretase (2, 3). APP mutations within the Aβ sequence (e.g. the Arctic and Iowa mutations) cause severe cerebral amyloid angiopathy or Alzheimer disease (AD) (4–8) by enhancing the tendency of the Aβ peptide to aggregate (7, 9) or by increasing its resistance to proteolytic degradation (10, 11). Mutations near the γ-secretase cleavage site such as the London (V717I, V717G), Indiana (V717F), and Florida (I716V) mutations lead to an increase in the amyloidogenic Aβ1-42 (12–17).

Amyloid precursor protein has been shown to be cleaved by γ-secretase through a series of sequential cleavage steps. First, there is ε-cleavage near the membrane-cytosol boundary, followed by γ-cleavage in the middle of the transmembrane domain (18). The ε-cleavage results in the release of an APP intracellular domain (AICD), whereas the γ-cleavage results in the generation of Aβ peptides (19–21). Several Aβ species consisting of 36 to 43 residues are generated and constitutively secreted. Aβ40 is the most predominant species, and although Aβ42 is a minor one, it predominates in the diffuse and mature plaques found in AD (22).

Most studies on APP mutations near γ-secretase cleavage site have focused on the ratio Aβ42/40 as the main mechanism by which these mutations exert their pathogenic effects (15, 17, 23). Here, we describe the clinical, neuropathologic, and biochemical characteristics of the recently described APP I716F mutation (24). This family shows the youngest age of onset for this locus, an aggressive clinical course, and severe neuropathologic phenotype. Biochemical experiments confirmed a marked increased in Aβ42/40 ratio and reduced APP proteolysis by γ-secretase.

MATERIALS AND METHODS
Neuropathologic Examination
The neuropathologic study was performed on formalin-fixed, paraffin-embedded samples, as previously described (25). Sections of frontal (Area 8), primary motor, primary
sensory, parietal, temporal superior, temporal inferior, anterior cingulate, anterior insular, and primary and associative visual cortices, entorhinal cortex and hippocampus, caudate, putamen and pallidum, medial and posterior thalamus, sub-thalamus, nucleus basalis of Meynert, amygdala, midbrain (2 levels), pons, medulla oblongata, cerebellum cortex, and dentate nucleus were examined. Dewaxed sections (5-μm thick) were stained with hematoxylin and eosin and with Klüver-Barrera, or were processed for immunohistochemistry using the EnVision+ system peroxidase procedure (DAKO, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with 1 of the primary antibodies at 4°C overnight. Antibodies to glial fibrillary acidic protein (DAKO), Aβ (Boehringer, Barcelona, Spain), and ubiquitin (DAKO) were used at dilutions of 1:250, 1:50, and 1:200, respectively. Antibodies to Aβ1-40 and Aβ1-42 (a generous gift from Dr. Sarasa, Zaragoza, Spain) were used at dilutions of 1:50. Antibodies to α-synuclein (Chemicon, Barcelona, Spain) were used at a dilution of 1:3000. Monoclonal anti-phospho-tau AT8 (Innogeneics, Gent, Belgium) was diluted 1:50. Phospho-specific tau rabbit polyclonal antibodies Thr181, Ser199, Ser202, Ser231, Ser262, Ser396, and Ser422 (all from Calbiochem, Torrey Pines, CA) were used at a dilution of 1:100 except for anti-phospho-tauThr181, which was used at a dilution of 1:250. Antibodies to 3R and 4R tau (Upstate, Millipore, Barcelona, Spain) were used at dilutions 1:800 and 1:50, respectively. TAR DNA binding protein was studied by using a mouse monoclonal antibody (Abnova, TebuBio, Barcelona, Spain) at a dilution of 1:1000, and a rabbit polyclonal antibody (Abcam, Cambridge, UK) at a dilution of 1:2000. Phospho–TAR DNA binding protein was studied by using a mouse monoclonal antibody at a dilution of 1:5000 and a rabbit polyclonal antibody at a dilution of 1:2500 (both from Cosmo Bio Co., Ltd., Koto-ku, Japan). The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% H2O2. Sections were counterstained with hematoxylin. Sections processed for phospho-tau immunohistochemistry were boiled in citrate buffer before incubation with the primary antibody. Sections processed for Aβ and α-synuclein were pretreated with 95% formic acid.

Cell Culture and Transfections

Chinese hamster ovary (CHO) cells were cultured in Dulbecco’s Modified Eagle Medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum at 37°C with 5% CO2 in a tissue culture incubator. Cells were transfected using Fugene reagent (Invitrogen) according to the manufacturer’s instructions.

Plasmid Construction

Mutated cDNA constructs encoding APP V717I and APP I716F were introduced in human wild-type APP 695 cDNA by site-directed mutagenesis (Stratagene, Cedar Creek, TX).

Aβ Enzyme-Linked Immunosorbent Assay

Conditioned medium was collected 24 hours after transfection. Human Aβ1-40 was measured by ELISA, as described (26). Briefly, antibody 6E10 (against Aβ1-17; Chemicon, Temecula, CA) was used as a capture antibody and a rabbit polyclonal Aβ1-40 (Chemicon) as a detection antibody. After incubation for 3 hours, wells were washed with PBS and a horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson Laboratories, West Grove, PA) was added. Wells were washed with PBS, QuantaBlue reagent (Pierce, Rockford, IL) was added, and samples were read at 320 nm using a Victor3 Wallac plate reader (Perkin-Elmer, Waltham, MA). Human Aβ1-42 and Aβ1-x (as a measure of total Aβ) were detected using sensitive ELISA kits (Wako, Osaka, Japan, and IBL, Hamburg, Germany, respectively).

Membrane Preparations and Cell-Free AICD Generation Assay

For Western blot analysis of the APP C-terminal fragments (CTFs), cellular membranes were isolated from CHO

FIGURE 1. Pedigree and clinical characteristics of the family with the amyloid precursor protein (APP) I716F mutation. (A) Pedigree of the family: males are represented by squares, females by circles, shaded symbols indicate affected individuals, diagonal bands indicate deceased individuals. Ages at onset are shown below symbols. Ages at death are shown in italics below ages at onset. A blood sample was taken from the proband (II.I) for DNA analysis. (B) Axial and coronal 99mTc-Hexamethylpropyleneamine oxime single-photon emission computerized tomography of the proband (II.I) at age 33 years showing marked hypoperfusion (depicted in green) predominantly in parietal regions. (C) Proband’s DNA sequence of APP exon 17 showing a missense mutation (A→T) at the first position of Codon 716, which predicts an isoleucine-to-phenylalanine substitution (p.APP I716F).
cells transfected with wild-type, V717I, or I716F APP constructs, as described (27). For the brain samples, 100 to 200 mg of tissue was homogenized with the Proteo Extract Native Membrane Protein Extraction Kit (Calbiochem). Amyloid precursor protein intracellular domain was generated in vitro from membrane preparations of transfected CHO cells, as described (19). The samples were electrophoresed in 5% to 16% Tris-Tricine gels, transferred to 0.2-μm nitrocellulose membranes, and detected by immunoblotting with a rabbit anti-APP C-terminal (Sigma-Aldrich, St. Louis, MO) antibody.

**γ-Secretase Activity**

γ-Secretase activity in cell lysates was measured by a fluorometric activity Kit (R&D Systems, Minneapolis, MN).

**Statistical Analysis**

One-way analysis of variance was performed to analyze differences in Aβ levels, APP CTFs, and γ-secretase activity, followed by least significant difference post hoc analysis. Levene test was also performed to determine whether variances were equal.

**RESULTS**

**Family History**

The proband was a 33-year-old man who complained of a progressive history of forgetfulness and difficulties in concentrating, with the onset of symptoms at age 31 years (Fig. 1A). He had problems in remembering recent events and with abstract reasoning. Over the next 2 years, he showed...
difficulties planning, using utensils, and performing fine-hand sequences that interfered with his work as a gardener. He also displayed difficulties with the sense of direction, and as a result, he had to stop working and driving. Occasionally, he complained of irregular jerks in both arms. Neuropsychologic evaluation at the age of 33 years showed deficits in verbal and visual memory, attention, and calculating with marked motor and constructive apraxia. His Mini Mental State Examination score was 21 of 30. Brain magnetic resonance imaging at the age of 33 years showed bilateral atrophy in frontoparietal regions. 99mTc-Hexamethylpropyleneamine oxime brain perfusion single-photon emission computerized tomography showed hypoperfusion in both parietal regions (Fig. 1B). The patient continued to worsen and died at the age of 36 years. The father’s proband had developed dementia at the age of 35 years. A brain biopsy at the age of 39 years showed abundant diffuse and neuritic plaques, amyloid angiopathy, and neurofibrillary tangles immunoreactive for phosphorylated tau. The diagnosis made was AD, and he died at the age of 41 years. We were unable to investigate family history of dementia because he had been adopted. The proband’s sibling is cognitively normal and refused genetic testing. There was no other family history of dementia. Genetic screening of the coding regions of PSEN1, PSEN2, and APP genes in the proband disclosed an APP I716F mutation (Fig. 1C; [24]). APOE genotype was ε3ε3.

Neuropathologic Examination of the Proband

Neuropathologic evaluation showed global cerebral atrophy. Microscopic examination revealed extensive diffuse and neuritic perineuronal Aβ plaques, subpial deposits, and cerebral amyloid angiopathy. Neuritic plaques, composed mainly of Aβ1-40 and Aβ1-42, predominated in the entorhinal cortex, subiculum, hippocampus, amygdala, and inner region of the temporal and orbitofrontal cortex (Fig. 2A–C). In contrast, diffuse plaques, perineuronal plaques, and subpial Aβ deposits were mainly stained with antibodies to Aβ1-42 (Fig. 2D–F) and were present in most of the neocortex. Cerebral amyloid angiopathy affected small arteries, arterioles, and venules of the meninges and the brain (Fig. 2B); capillaries were spared. Neurofibrillary tangles and neuropil threads were present in large numbers in the entorhinal and transentorhinal cortices, hippocampus, amygdala, nucleus basalis of Meynert, septal nuclei, and the entire cerebral neocortex, including the primary sensory and motor areas (Fig. 2G, H). Neurofibrillary tangles were also present in selected nuclei of the brainstem, including the substantia nigra, motor ocular nuclei, locus ceruleus, and reticular formation of the pons and medulla oblongata. Dystrophic neurites containing hyperphosphorylated tau were abundant in the amygdala, hippocampus, and entorhinal cortex, corresponding to AD Stage VI of Braak. All of these structures were stained with AT8, other phospho-specific anti-tau antibodies, and with anti-4R (Fig. 2G), anti-3R (Fig. 2H), and anti-ubiquitin antibodies.

α-Synuclein immunoreactivity was observed in the amygdala in the form of large numbers of Lewy bodies and aberrant neurites (Fig. 2I). TAR DNA binding protein–immunoreactive inclusions were absent. Neuronal loss, astrocytic gliosis, and microgliosis were moderate in the cerebral neocortex but were more marked in the entorhinal and perirhinal cortex, subiculum, and amygdala. Astrocytes predominated in the inner cortical layers of the neocortex, plexiform layers of the hippocampus, and around neuritic plaques. Likewise, microglia were increased in number in the cerebral cortex and white matter and more abundantly around neuritic plaques.

Biochemical Effects of the APP I716F Mutation

We next investigated the effects of this mutation on APP processing in transfection studies using CHO cells. Quantitative analysis of the major Aβ species in the conditioned medium from APP I716F–transfected CHO cells showed increased (~2-fold) Aβ42, reduced (~0.5-fold) Aβ40, and total Aβ (~0.5 fold) levels compared with that of wild-type APP. Similar but milder results were found in APP V717I–transfected CHO cells.

![Figure 3](http://jnen.oxfordjournals.org/)

**FIGURE 3.** Amyloid-β (Aβ) levels from Chinese hamster ovary (CHO) cells transfected with the APP I716F and V717I mutations. (A) Amyloid-β40, Aβ42, and Aβ1-42 (total) levels from CHO cells transfected with the APP I716F and V717I mutations. Amyloid-β was measured by ELISA in the conditioned medium 24 hours posttransfection. Treatment with N-[3,5-difluorophenyl]acetyl]-l-alanyl-2-phenylglycine e-1,1-dimethylethyl ester, a potent inhibitor of γ-secretase, was used as a positive control in wild-type APP-transfected cells. Amyloid precursor protein I716F–transfected CHO cells showed increased (~2 fold) Aβ42, reduced (~0.5 fold) Aβ40, and total Aβ (~0.5 fold) levels compared with that of wild-type APP. Similar but milder results were found in APP V717I–transfected cells. *, One-way analysis of variance, p < 0.05 vs wild-type APP. Average Aβ levels are expressed as a percentage relative to wild-type. Values represent the mean ± SD of 3 independent experiments. (B) Amyloid-β42/40 ratios were calculated for wild-type APP and the APP mutations. Results are expressed relative to wild-type. Amyloid-β42/40 ratio was markedly increased in cells transfected with the APP I716F or V717I mutations. *, One-way analysis of variance, p < 0.05 vs wild-type APP.
and total Aβ (~0.5-fold) levels compared with those of wild-type APP (Fig. 3A). As a result, the Aβ_{32/40} ratio was markedly increased (~4-fold) in cells transfected with the APP I716F mutation (Fig. 3B). Results were compared with cells transfected with the adjacent APP V717I mutation (13), which led to similar but milder effects. Membrane preparations from APP I716F- and V717I-transfected cells showed a marked increase (2- and 2.5-fold) in APP CTFs compared with that of wild-type APP (Fig. 4A). Interestingly, the levels of APP CTFs were higher in the proband’s brain homogenates.

**FIGURE 4.** Effects of amyloid precursor protein (APP) I716F mutation on APP processing. (A) Effects on APP C-terminal fragments (CTFs). Immunoblotting from membrane preparations from Chinese hamster ovary (CHO) cells transfected with the I716F and V717I mutations. Amyloid precursor protein I716F and V717I transfected cells showed marked accumulation of APP CTFs. Treatment with the γ-secretase inhibitor N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-dimethyllethyl ester was used as a positive control on wild-type APP-transfected cells. Data are expressed relative to wild-type (wt) and represent mean ± SD of 3 experiments. Quantitation is shown below. *, One-way analysis of variance, p < 0.05 vs wild-type APP. (B) Western blot of APP CTFs from brain homogenate samples of the proband (1716F), young healthy controls (C1, C2), and late-onset Alzheimer disease (AD1-3) patients. Amyloid precursor protein CTFs are higher in the proband’s brain homogenates than in sporadic AD patients or young healthy controls. Quantification of the ratio CTFs/full-length APP is shown below. The proband is indicated as a gray diamond. (C) Amyloid precursor protein intracellular domain (AICD) was measured in a cell-free assay from CHO cells transfected with wild-type APP or the APP I716F and V717I mutations. Lysates from APP I716F and V717I transfected cells showed reduced AICD generation. Lysates kept at 4°C or treated with N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycin e-1,1-dimethyllethyl ester served as controls. Data are expressed relative to wild-type and represent mean ± SD of 3 experiments. (D) γ-Secretase activity was measured by a fluorometric assay in cell lysates from cells transfected with the APP I716F and V717I mutations. Cells transfected with APP I716F and V717I mutations showed reduced fluorogenic activity. Values are expressed as a percentage of wild-type and are the average of 2 independent experiments. *, One-way analysis of variance, p < 0.05 vs wild-type APP.
than in sporadic AD patients or young and elderly healthy controls (Fig. 4B). Incubation of membrane preparations from APP I716F- and V717I-transfected cells showed a reduced production of AICD and accumulation of CTFs (Fig. 4C). Finally, lysates from cells transfected with APP I716F or V717I mutations showed reduced γ-secretase activity assessed by a fluorogenic kit assay (Fig. 4D).

DISCUSSION

The APP I716F mutation had previously been described as an artificial mutation with extreme effects on the Aβ42/40 ratio (23, 28–30). Here, we describe the full clinical, neuropathologic, and biochemical profile of the recently reported APP I716F mutation (24). This FAD mutation is associated with the youngest age of onset for this locus (mean age of onset, 49 years [31]), supporting the strong inverse association between Aβ42/40 ratio and age of onset (17). Neuropathologic study of the proband’s brain revealed atypical generalized and extensive Aβ deposition in the brain and cerebral blood vessels. Amyloid-β deposition formed neuritic plaques in the inner regions of the temporal lobe and large numbers of Aβ42-predominant diffuse plaques, perineuronal plaques, and subpial deposits in the neocortex. As in other families with APP mutations (32, 33), as well as in sporadic AD cases (34), Lewy bodies were observed in the amygdala, suggesting that α-synuclein pathology is downstream of Aβ42 deposition in these families. However, the reasons for such selective involvement are not known.

Biochemical characterization of this mutation extended previously described results (23, 28–30). The APP I716F mutation leads to a marked increase in Aβ42 and the Aβ42/40 ratio and reduced Aβ40 and Aβ1–40 levels. As expected, the Aβ42/40 ratio was lower than other studies that used APP C99 (which is the direct substrate for γ-secretase) for transfection studies (23, 28, 29). As for other APP mutations located near the γ-secretase cleavage site (17, 35), we also showed that the APP I716F mutation led to a prominent accumulation of APP CTFs in transfected cells. Interestingly, APP CTFs were higher in the proband’s brain homogenates than in sporadic AD patients and healthy controls. Although the availability of only a single brain sample precluded an in-depth analysis of APP processing in this kindred, this increase suggests that APP CTF accumulation is not an artifact in the cellular model. However, the increase in APP CTFs does not necessarily reflect reduced γ-secretase activity because it has been observed without concomitant reduction in Aβ secretion (36). Therefore, we cannot completely exclude the possibility that this accumulation may be partially due to impaired degradation. In any case, accumulation of APP CTFs has been shown to be neurotoxic and to cause neurodegeneration in vivo and in vitro (35, 37, 38), and we cannot exclude the possibility that this can also contribute to the neurodegeneration observed in this family.

We further demonstrated a reduced AICD generation in cells expressing the APP I716F mutation. Amyloid precursor protein intracellular domain results from the ε-cleavage of APP β-CTF that occurs near the membrane-cytoplasm boundary (19–21). This initial cleavage is followed by different γ-cleavage events toward the middle of the transmembrane domain to generate different Aβ species (18). The presence of cleavage sites at every 3 residues between the γ- and ε-cleavage fits well with an α-helical model (39–42). According to this model, the cleavage sites for Aβ49, Aβ46, Aβ43, and Aβ40 are aligned on the α-helical surface of the β-CTF molecule, whereas those for Aβ48, Aβ45, and Aβ42 are aligned on the other α-helical surface (39). Although we only measured the major secreted Aβ species, the reduction in γ- and ε-cleavage and APP CTF accumulation in our study suggests that the APP I716F mutant is poorly processed by γ-secretase. This is supported by a reduced activity measured by a fluorogenic assay. Taken together, our results suggest that a selective loss of function in APP proteolysis can be caused by an APP mutation. A similar loss-of-function mechanism has been proposed for PSEN mutations (43). Consistent with this notion, a FAD-associated PSEN1 mutation was shown to slow sequential intramembrane cleavage by γ-secretase and other GXXG-aspartyl proteases, resulting in longer cleavage products (44).

Overall, this family reveals that, although the Aβ42/40 ratio seems to be the best indicator of severity of the disease in patients with APP mutations, the reduced processing might also contribute to the disease process and suggests an additional mechanism by which some mutations around the γ-secretase cleavage site may lead to AD.

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REFERENCES


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