Genetic risk and transcriptional variability of amyloid precursor protein in Alzheimer’s disease

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It is well established that Alzheimer’s disease causing mutations in APP, PSEN1 and PSEN2 lead to a relative increased production of Aβ42, thereby fostering its deposition in plaques. Recently others and we showed that amyloid precursor protein (APP) overproduction, either as a result of genomic locus duplication or altered regulatory sequences in the APP promoter region, leads to early-onset disease. Here, we have expanded our study of genetic variability in the APP promoter to a large group of well-documented Belgian patients (n = 750, mean onset age = 75.0 ± 8.6, range = 37–96). We identified three different APP promoter mutations (−369C→G, −534G→A and −479C→T) in seven patients. In patients with onset age ≥70 years (n = 204), we identified one patient carrying the London APP V717I mutation while no patients carried an APP locus duplication, indicating that APP promoter mutations (n = 2) were more frequently associated with increased risk for early-onset Alzheimer’s disease. The two mutations (−369C→G and −534G→A) increasing APP promoter activity by nearly 2-fold and mimicking an APP duplication, appeared in probands of families with multiple patients with dementia. The −479C→T mutation that increased APP expression only mildly (1.2-fold), was observed in four patients with onset ages ranging from 62 to 79 years (mean 71.5 years), suggesting that its contribution to disease risk is more pronounced at later age due to modulating factors. In conclusion, we provided evidence that mutations in APP regulatory sequences are more frequent than APP coding mutations, and that increased APP transcriptional activity constitutes a risk factor for Alzheimer’s disease with onset ages inversely correlated with levels of APP expression.

Keywords: Alzheimer disease; risk factor; amyloid precursor protein; promoter; mutations

Abbreviations: AAO = age at onset in patients; Aβ = amyloid β; APP = amyloid precursor protein; MAQ = multiplex amplicon quantification; MMSE = Mini-Mental State Examination; SPECT = single photon emission computed tomography; STR = short tandem repeat

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Introduction

Alzheimer’s disease is a progressive neurodegenerative brain disorder and the most frequent cause of dementia in the elderly. Pathologically, the disease is characterized by the formation of two distinct brain lesions i.e. parenchymal amyloid plaques consisting mainly of aggregated and deposited amyloid β (Aβ) peptides, and intraneuronal neurofibrillary
Together our data supported a 1.2% allelic variability of the APP proximal promoter in patients with onset ages ≤70 years. Also, four of five mutations were located in a 200 bp fragment (−540 to −340) that was highly conserved between species (>95%). Here we extended our studies of APP promoter genetic variability in risk for Alzheimer’s disease in the Belgian series of patients independently of onset age.

We provide genetic and clinical data obtained in a total of 750 patients with mean onset age of 75.0 ± 8.6 years ascertained in the frame of a prospective study of dementia in Flanders, the Dutch speaking region of Belgium (Engelborghs et al., 2003, 2006a).

Material and methods

Study groups

Belgian Alzheimer’s disease patients (n = 750) were systematically ascertained in the frame of a large prospective study of neurodegenerative and vascular dementia in Flanders (Table 1), the Dutch-speaking region of Belgium (Engelborghs et al., 2003, 2006a). In a previous systematic study of the APP promoter we had included 180 of the 204 patients with onset age <70 years (Table 1), and identified three patients that carried genetic variants (−479C→T, −571G→A and −369C→G). Here we have included detailed clinical data of two patients, d1081 and d5165, who carried a mutation affecting APP expression in vitro (Table 2). All patients were examined at the Memory Clinic of Middelheim General Hospital, Antwerpen, Belgium (P.P.D.D. and S.E.). Each patient underwent a diagnostic neuropsychological examination, including Mini-Mental State Examination (MMSE) (Folstein et al., 1975), structural neuroimaging consisting of brain CT and/or MRI and functional neuroimaging [single photon emission computed tomography (SPECT)]. Consensus diagnosis of possible or probable Alzheimer’s disease was given by at least two neurologists based on the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer’s Disease and Related Disorders Association (NINCDS/ADRDA) criteria (McKhann et al., 1984). For all patients, detailed data on family history of dementia in first-, second-, and third-degree relatives were collected by interviewing the caregiver, mostly a next of kin of the patient. Here we considered the disease familial when the patient had one or more first-degree relatives with dementia. CSF was sampled in a subset of patients (n = 234), and levels of β-amyloid peptide (Aβ1–42), total tau protein (tau) and tau phosphorylated at threonine 181 (P-tau181P) were determined blinded and in duplicate, with commercially available single parameter ELISA kits (Innogenetics, Gent, Belgium) as described previously (Engelborghs et al., 2006b). As a result of follow-up studies, autopsy was performed in 59 patients. All autopsied patients (59/59)

Table 1 Characteristics of the Belgian study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>Total AD group</th>
<th>AD (AAO ≤ 70 years)</th>
<th>AD (AAO &gt; 70 years)</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>750</td>
<td>204</td>
<td>546</td>
<td>435</td>
</tr>
<tr>
<td>Mean AAO/AAI ± SD (years)</td>
<td>75.0 ± 8.6</td>
<td>64.0 ± 6.0</td>
<td>79.1 ± 5.2</td>
<td>59.6 ± 15.2</td>
</tr>
<tr>
<td>Mean AAO/AAI range (years)</td>
<td>37–96</td>
<td>37–70</td>
<td>71–96</td>
<td>37–92</td>
</tr>
<tr>
<td>Females (%)</td>
<td>66.4</td>
<td>55.4</td>
<td>70.5</td>
<td>56.1</td>
</tr>
<tr>
<td>Familial AD (%)</td>
<td>22.3</td>
<td>39.2</td>
<td>15.9</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

AAI = age at inclusion for control individuals; n.a. = not applicable.
fulfilled the criteria for definite Alzheimer’s disease. The Belgian control group consisted of 435 healthy Dutch-speaking Belgian individuals and included subjects (n = 252) without neurological or psychiatric antecedents or with neurological complaints without organic disease involving the central nervous system, of which 195 individuals had normal scores on the MMSE (Engelborghs et al., 2003). The other 183 subjects were selected among married- in individuals in families with neurological diseases collected for genetic linkage studies. Genomic control using 31 randomly selected microsatellite markers excluded population stratification in the control group. Further characteristics of the patient and control groups are shown in Table 1.

After informed consent, blood samples of each proband were collected for genetic studies. In case of a positive family history, probands or their legal representatives were contacted by a research nurse for detailed genealogical studies. Blood samples were taken upon informed consent of the patient for DNA extraction, EBV cell lines, plasma and serum. Subject’s consent was obtained according to the Declaration of Helsinki (BMJ 1991; 302: 1194). All studies were approved by the medical ethical committee of the Middelheim General Hospital and the University of Antwerp.

**APP mutation analyses**

Mutation analyses of the APP proximal promoter were performed on genomic DNA extracted from whole blood DNA according to standard procedures. Mutation analysis of the −759/−258 fragment was performed using bi-directional sequencing. The APP −118C→A transversion was genotyped through one-directional sequencing of the −573/−25 fragment. Mutation analysis of APP was performed in the 204 patients with onset age ≤70 years (Table 1) by bi-directional sequencing of PCR amplicons of exons 16 and 17 of APP (Sleeegers et al., 2004).

Genomic DNA was PCR amplified using individually optimized reaction conditions. PCR amplicons were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH, USA) and sequenced using PCR primers and the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s recommendations. Labelled PCR fragments were separated on an Applied Biosystems 3730 DNA analyser (Applied Biosystems).

**APP dosage analysis**

**Quantitative real-time PCR**

APP copy number was quantified using SYBR Green I based real-time PCR (rtPCR) assays developed with the Primer Express software (Applied Biosystems) for APP exons 5, 11 and 18 as well as for two control genes, ubiquitin C (UBC) and β2-microglobulin (B2M). Genomic DNA, in quantities of 20 ng, were rtPCR amplified in 30 µl containing 1 × qPCR™ Mastermix Plus for SYBR® Green I [without uracil-N-glycosylase (UNG); Eurogentec, Seraing, Belgium] and 12 pmol of each primer on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using a universal amplification protocol (Applied Biosystems). A dissociation curve analysis was performed to exclude non-specific amplification. APP test amplicons were normalized for the two control amplicons. The dosage quotient (DQ), representing the ratio of normalized APP quantities of patients versus control individuals, were calculated and were considered indicative for the presence of an APP locus duplication when >1.3.

**Multiplex amplicon quantification**

Copy numbers of APP and 11 surrounding genes were determined using a technique for multiplex amplicon quantification (MAQ). The MAQ technique involves the quantification of fluorescently labelled test amplicons and control amplicons that were obtained in one multiplex PCR (mPCR) reaction. Primers are designed to allow simultaneous amplification of test and control fragments under the same mPCR conditions. Test amplicons are located in the area being examined for gene dosage while control amplicons are located on other chromosomes. PCR fragments are resolved by fluorescent fragment analysis on an Applied Biosystems 3730 DNA analyser (Applied Biosystems). Peak areas of the test amplicons are normalized based on the peak areas obtained for control amplicons and a DQ is calculated from the normalized peak areas. The APP MAQ assay we designed to detect APP duplications consisted of 5 test amplicons located in APP, 17 test amplicons located in 11 surrounding genes (ADAMTS1, ADAMTS5, BACH1, c21orf42, CCT8, CYYR1, FLJ42200, GRIK1, JAM2, MRPL39 and USP16) and 15 control amplicons located on different chromosomes. The 37 amplicons were obtained by PCR amplification on 50 ng genomic DNA in one mPCR reaction with a total volume of 15 µl containing 1 × Titanium™ Taq PCR Buffer (Clontech, Palo Alto, CA, USA), 0.5 × Titanium™ Taq DNA Polymerase (Clontech) and optimized primer mix. DQs were calculated using the MAQ software (MAQs) package (http://www.vibgeneticservicefacility.be/MAQ.htm). DQ values >1.3 were considered indicative of a genomic duplication of test amplicons.

**APP allele sharing analyses**

In patients carrying the same mutation, we examined a potential genetic relationship by genotyping seven short tandem repeat (STR) markers located within the APP locus from 281 kb upstream to 16 kb downstream of APP. Three markers were selected from the Marshfield gender-averaged genetic map (D21S1253, D21S1443 and D21S1896). The remaining four markers were identified with the Tandem Repeat Finder program (Benson, 1999). Chr21rep5 is located in AP000228.1 starting at nt 66052, Chr21rep6 in AP001442.1 at nt 41663, Chr21rep7 in AP001439.1 at nt 70766 and Chr21rep9 in AP001596.1 at nt 43094.

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**Table 2 APP proximal promoter mutations in Belgian Alzheimer’s disease patients**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Patient ID</th>
<th>Onset age (years)</th>
<th>APOE genotype</th>
<th>F/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>−369C→G</td>
<td>d1081*</td>
<td>61</td>
<td>4/4</td>
<td>F</td>
</tr>
<tr>
<td>−479C→T</td>
<td>d5165*</td>
<td>62</td>
<td>3/3</td>
<td>–</td>
</tr>
<tr>
<td>−369C→G</td>
<td>d4550</td>
<td>78</td>
<td>3/4</td>
<td>F</td>
</tr>
<tr>
<td>−479C→T</td>
<td>d1883</td>
<td>74</td>
<td>3/3</td>
<td>–</td>
</tr>
<tr>
<td>−369C→G</td>
<td>d1908</td>
<td>79</td>
<td>2/3</td>
<td>–</td>
</tr>
<tr>
<td>−479C→T</td>
<td>d4587</td>
<td>71</td>
<td>3/4</td>
<td>–</td>
</tr>
<tr>
<td>−534G→A</td>
<td>d4904</td>
<td>77</td>
<td>4/4</td>
<td>F</td>
</tr>
</tbody>
</table>

*The APP promoter mutations in probands d1081 and d5165 were identified in a previous study of patients with onset age ≤70 years (Theuns et al., 2006). F = patients with a first degree family history of Alzheimer’s disease.
Using fluorescently labelled primers and individually optimized reaction conditions genomic DNA of 20 ng was PCR amplified. PCR products were resolved on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). Allele frequencies for each STR marker were estimated in 51 Belgian control individuals.

**APOE genotyping**

APOE genotyping was performed by pyrosequencing (PCR primers: 5’-CTGGGCGCGGACATGGAG-3’ and 5’-biotinylated-GGCCGGGGCTGTACAG-3’) and pyrosequencing primers: 5’-GGCGACATGGAGGA-3’ and 5’-ATGCGATGACCTGC-3’). Using a standard thermal cycling protocol and empirically determined reaction conditions 20 ng of genomic DNA was PCR amplified. Sequencing reactions were performed using the PSQ HS96 SNP Reagent Kit (Biotage, Uppsala, Sweden) on a PSQ HS96A System (Biotage) and analysed with the PSQ HS96 SNP Software (Biotage).

**Results**

**APP promoter genetic variability**

We examined the contribution of genetic variability in the APP proximal promoter to Alzheimer’s disease risk by direct sequence analysis of −573/−25 and −759/−258 PCR amplicons in 750 Belgian patients ascertained in a prospective study of dementia in Flanders, the Dutch speaking region of Belgium, and 435 control individuals (Table 1). The two genomic fragments comprise the four promoter mutations −118C→A, −369C→G, −479C→T and −534G→A, that were shown to affect APP transcriptional activity in vitro (Theuns et al., 2006). Two mutation carriers (−369C→G and −479C→T) had previously been identified among 180 patients with age at onset (AAO) ≤70 years belonging to this Belgian patient group (Table 2) (Theuns et al., 2006). Here, we identified an additional five carriers of an APP promoter mutation with late-onset Alzheimer’s disease (AAO >70 years, Table 2). Thus in total we detected in the Belgian group seven patients carrying one of three different heterozygous mutations (−369C→G, −479C→T and −534G→A) located in the −759/−258 PCR fragment of the APP proximal promoter (7/50 = 0.9%). All mutation carriers had a clinical diagnosis of probable Alzheimer’s dementia. Two patients had died (d1883 and d4587), however no autopsy was performed. All three mutations identified were among the four APP promoter mutations that increased APP transcriptional activity in vitro by a factor ranging from 1.2 to 1.8, and were associated with Alzheimer’s disease (Theuns et al., 2006). APOE genotypes of the mutation carriers are included in Table 2.

Three patients carried one of two mutations affecting APP expression significantly (−369C→G and −534G→A), and were the probands of families with multiple patients with dementia based on a family informant and medical records when available (Fig. 1). The remaining four patients carried the −479C→T mutation and had no first-degree relatives affected with dementia. Allele sharing analysis in the probands and available relatives using seven STR markers in the APP locus showed that the two patients (d1081 and d4550) with the −369C→G mutation shared alleles for five neighbouring markers (Chr21rep6, D21S1253, D21S1443, Chr21rep7 and Chr21rep9) spanning a region of ~460 kb, from 241 kb upstream to 219 kb downstream of the mutation. Sharing for these five markers was not observed in 51 control individuals, suggesting that the frequency of the shared haplotype is <1% and supporting a distant common founder for the −369C→G mutation. The four −479C→T carriers did not share common alleles at neighbouring STR markers, suggesting that this mutation resulted in each patient from a novel mutational event.

In the control individuals we observed two individuals carrying the −479C→T transition. Healthy −479C→T carriers were still at risk of developing Alzheimer’s disease since their AAI (ages at inclusion) were 38 and 51 years. One control individual, aged 76 years, carried the −369C→G mutation. The variants −343A→C and −375G→C that we had previously identified only in control individuals (Theuns et al., 2006), were not observed in the extended control group in the patients.

**Clinical features of familial APP −369C→G and −534G→A carriers**

Two Alzheimer’s disease probands (d1081 and d4550) carried the −369C→G transversion and one proband (d4904) the −534G→A transition that in vitro increased APP transcriptional activity by 1.8 and 1.7, respectively (Theuns et al., 2006, 2007). The patients had a clinical diagnosis of probable Alzheimer’s disease with a mean AAO 71 (range 62–83) years. Their AAO (range 31–66) years and their number of carriers were: Patients d1081 (2); d4550 (2) and d4904 (2). The mean number of carriers per family was 2.1 (range 1–2). The two −369C→G carriers had one common allele for seven STR markers spanning more than 340 kb (Chr21rep9) from 579 kb downstream to 219 kb upstream of the −369C→G mutation. Relatedness was confirmed using seven STR markers (Chr21rep6, D21S1253, D21S1443, Chr21rep7 and Chr21rep9) spanning a region of ~460 kb, from 219 kb upstream to 219 kb downstream of the mutation. Sharing for these five markers was not observed in 51 control individuals, suggesting that the frequency of the shared haplotype is <1% and supporting a distant common founder for the −369C→G mutation. The four −479C→T carriers did not share common alleles at neighbouring STR markers, suggesting that this mutation resulted in each patient from a novel mutational event.
In their families multiple patients with dementia were identified in 2–3 generations (Fig. 1). Patient d1081 had an onset age of 61 years and suffered from progressive memory problems and poor judgement, interfering with her social and professional activities. Her cognitive state deteriorated gradually, and at 70 years behavioural and psychiatric disturbances (visual hallucinations, anxiety, restlessness, aspontaneity and emotional bluntness) were noticed. At age 71, brain CT and SPECT were compatible with a clinical diagnosis of probable dementia of the Alzheimer type (Table 3). The neuropsychological examination demonstrated deficits in recent memory, orientation in time and space, concentration, visuoconstructive skills, word naming and verbal fluency. The profile of the CSF biomarker panel (Aβ1–42, tau, P-tau181P) was in support of a diagnosis of the disease. Family history showed that the father had suffered from cognitive deterioration at 90 years (Family DR39, Fig. 1). Moreover, 4 out of 13 of her siblings had dementia though at a later onset age, suggestive of genetic heterogeneity in this family.

Patient d4550 was 80 years when she was referred to the Memory Clinic because of progressively worsening disorientation and memory problems that were first noted by her relatives at age 78. Structural and functional neuroimaging were compatible with a clinical diagnosis of probable Alzheimer’s disease (Table 3). Neuropsychological examination revealed severe deficits in recent memory and deficits in long-term memory, visuoconstructive skills as well as a disorientation in time and a constructional apraxia. Family history was positive in the first degree, with both the mother and one sister of the patient reported to have suffered from the illness (Family DR89, Fig. 1).

Patient d4904 consisted of progressive memory deficits at age 77. At age 82, she presented with severe memory loss, word finding difficulties and behavioural disturbances (dissociation, irritability, and emotional bluntness). Presented symptoms of patient d4904 consisted of progressive memory deficits at age 77. At age 82, she presented with severe memory loss, word finding difficulties and behavioural disturbances (dissociation, irritability, and emotional bluntness).

Table 3 Clinical characteristics of APP promoter mutation carriers

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Mutation</th>
<th>Gender (f/m)</th>
<th>Onset age (years)</th>
<th>Current age or age at death† (years)</th>
<th>Age at clinical work-up (years)</th>
<th>MMSE score</th>
<th>Structural neuroimaging (CT/MRI)</th>
<th>Functional neuroimaging (SPECT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d1081*</td>
<td>−369C→G</td>
<td>f</td>
<td>61</td>
<td>75</td>
<td>71</td>
<td>10/30</td>
<td>Cortico-subcortical atrophy; PWML (CT)</td>
<td>Marked relative bilateral temporal and parietal HP, left &gt; right, extending to bifrontally, left &gt; right. Preserved perfusion of the sensorimotor cortex bilaterally. Scintigraphic indications of subcortical loss</td>
</tr>
<tr>
<td>d4550</td>
<td>−369C→G</td>
<td>f</td>
<td>78</td>
<td>84</td>
<td>80</td>
<td>19/30</td>
<td>Cortico-subcortical and cerebellar atrophy; PWML: old lacunar infarctions in the BG bilaterally (CT)</td>
<td>Relative bilateral frontal, temporal and parietal HP, right &gt; left. Preserved perfusion of the sensorimotor cortex bilaterally</td>
</tr>
<tr>
<td>d4904</td>
<td>−534G→A</td>
<td>f</td>
<td>77</td>
<td>85</td>
<td>82</td>
<td>8/30</td>
<td>Marked cortico-subcortical atrophy; PWML (MRI)</td>
<td>Marked relative bilateral frontal, frontoparietal and temporal HP, left &gt; right. Relatively preserved perfusion of the sensorimotor cortex bilaterally</td>
</tr>
<tr>
<td>d1883</td>
<td>−479C→T</td>
<td>m</td>
<td>74</td>
<td>83‡</td>
<td>80</td>
<td>11/30</td>
<td>Cortico-subcortical atrophy; PWML: old lacunar infarctions in the BG bilaterally (CT)</td>
<td>Marked relative bilateral temporal HP (left &gt; right), extending to the frontal and parietal areas bilaterally. Relatively preserved perfusion of the sensorimotor cortex bilaterally. Scintigraphic indications of subcortical loss</td>
</tr>
<tr>
<td>d1908</td>
<td>−479C→T</td>
<td>f</td>
<td>79</td>
<td>86</td>
<td>83</td>
<td>22/30</td>
<td>Cortico-subcortical atrophy and PWML, normal for age (CT)</td>
<td>Relative bilateral frontal, temporal and parietal HP, left &gt; right. Preserved perfusion of the sensorimotor cortex bilaterally. Cortico-subcortical atrophy and PWML, normal for age (B)</td>
</tr>
<tr>
<td>d4587</td>
<td>−479C→T</td>
<td>f</td>
<td>71</td>
<td>80†</td>
<td>74</td>
<td>22/30</td>
<td>Cortico-subcortical atrophy and PWML, normal for age (MRI)</td>
<td>Relative bilateral frontal, and parietal HP extending to the frontal and parietal areas bilaterally</td>
</tr>
<tr>
<td>d5165*</td>
<td>−479C→T</td>
<td>f</td>
<td>62</td>
<td>69†</td>
<td>63</td>
<td>22/30</td>
<td>Cortico-subcortical atrophy, normal for age (MRI)</td>
<td>Relative bilateral temporal HP, extending to the frontal and parietal areas bilaterally</td>
</tr>
</tbody>
</table>

PWML = periventricular white matter lesions; BG = basal ganglia; HP = hypoperfusion.

The APP promoter mutations in probands d1081 and d5165 were identified in a previous study of Alzheimer’s disease patients with onset age equal to or less than 70 years (Theuns et al., 2006).
agitation, restlessness and verbal aggression). Brain MRI and SPECT supported the clinical diagnosis of probable Alzheimer’s disease (Table 3). Neuropsychological examination showed severe recent memory deficits, decreased verbal fluency, impaired visuconstructive skills and disorientation in time and space and poor concentration. Frontal disinhibition was also noticed upon examination. The profile of the CSF biomarker panel (\(A\beta_{1-42}, \tau, P-\tau_{181P}\)) was in support of a clinical diagnosis of probable Alzheimer’s disease. Family history was positive with a paternal aunt and the paternal grandmother reported to have suffered from dementia (Family DR86, Fig. 1). Her at-risk father had died of cancer at age 64.

Clinical features of sporadic \(APP–479C\rightarrow T\) mutation carriers

Four probands carried the \(-479C\rightarrow T\) mutation that increased \(APP\) transcriptional activity \textit{in vitro} by a factor 1.2 (Theuns \textit{et al.}, 2006) (Table 2). Their onset ages varied from 62 to 79 years, and two patients died without autopsy.

Patient d1908 was hospitalized at 83 years, 4 years after onset of the first symptoms that consisted of progressively worsening memory problems. Structural and functional neuroimaging were compatible with a clinical diagnosis of probable Alzheimer’s disease (Table 3). Neuropsychological examination showed deficits in recent memory, concentration, calculia, ideational praxis, word naming and concentration. The profile of CSF tau and \(A\beta_{1-42}\) levels was compatible with the disease according to the discrimination line described by Hulstaert \textit{et al.} (1999). The clinical diagnosis of probable Alzheimer’s disease was furthermore supported by neuropsychological retesting at age 84 and 85. Family history revealed an aunt of the patient with dementia, but no first-degree relatives.

Patient d1883 had an onset age of 74 years; presenting symptoms of progressive memory deficits and disorientation in time. Clinical and neuropsychological examination at age 78 revealed a MMSE score of 18/30 with severe memory disturbances, disorientation in time and space and behavioural disturbances (mainly aggressiveness). At age 80, the patient was hospitalized because of an intolerable living situation at home due to severe dementia and aggressiveness. The clinical neurological examination displayed orobuccal dyskinesias and extrapyramidal signs that were probably due to pharmacological drug treatment. Structural and functional neuroimaging was compatible with a diagnosis of probable Alzheimer’s disease (Table 3). Neuropsychological examination showed that memory, orientation in time and space, concentration, problem solving and ideomotor praxis were severely impaired. The patient died at age 83, 9 years after disease onset. An autopsy was not performed.

Patient d4587 was referred to the Memory Clinic at age 74. She suffered from progressively worsening memory problems over the last 3 years. Neuropsychological examination showed deficits in recent memory, concentration, calculia and disorientation in time and space whereas praxis and gnosis were normal. Structural and functional neuroimaging was compatible with a clinical diagnosis of probable Alzheimer’s disease (Table 3). The patient also exhibited symptoms of an associated depression. Family history showed that the grandfather and two nephews suffered from dementia as well.

Patient d5165 was referred to the Memory Clinic at age 63 after 1 year of progressively worsening (recent) memory problems. Brain CT and SPECT were compatible with a diagnosis of probable Alzheimer’s disease (Table 3). Neuropsychological investigation showed deficits in recent memory, problem solving, calculia, ideational praxis, word naming and concentration. The CSF biomarker panel (\(A\beta_{1-42}, \tau, P-\tau_{181P}\)) profile and clinical follow-up with neuropsychological retesting and neuroimaging at age 66 were in support of the clinical diagnosis of probable Alzheimer’s disease. The patient died at age 69; autopsy was not performed.

\textbf{APP mutations and duplications}

In the 204 Belgian Alzheimer’s disease patients with AAO \(\leq 70\) years we tested for the presence of missense mutations in \textit{APP} and for the \textit{APP} locus duplication. We identified only one missense mutation (1/204 = 0.5%), namely the London \textit{APP} V717I mutation (Goate \textit{et al.}, 1991), in a female proband with probable Alzheimer’s disease and onset age 64 years. The patient died at age 75 and autopsy showed typical neuropathology for the disease. The pedigree of the family is consistent with Mendelian inheritance of early-onset Alzheimer’s disease with a sibship consisting of three patients and two at-risk individuals. Molecular DNA diagnosis identified the mutation in one affected sister and one of two at-risk siblings. In all 204 patients we excluded the presence of an \textit{APP} locus duplication.

\textbf{Discussion}

We analysed the contribution of genetic variability in \textit{APP} to risk for Alzheimer’s disease in 750 patients that were recruited based on a standardized inclusion protocol comprising clinical examinations, neuropsychological testing and structural and functional imaging. Mean onset age in this group was 75.0 \pm 8.6 years (range 37–96 years), with 660 patients diagnosed with probable and 31 with possible and 59 with definite Alzheimer’s disease. In 180 of the 204 patients with onset age \(\leq 70\) years, we previously identified two patients, d1081 and d5165 (Table 2), carrying a mutation in the \textit{APP} proximal promoter (\(-369C\rightarrow G\) and \(-479C\rightarrow T\)) that increased \textit{APP} transcriptional activity \textit{in vitro} with a factor 1.8 and 1.2, respectively (Theuns \textit{et al.}, 2006). Here we expanded our search for \textit{APP} promoter mutations to the whole Alzheimer’s disease group and identified another five mutation carriers of \(-369C\rightarrow G\) \((n = 1)\), \(-479C\rightarrow T\) \((n = 3)\) and \(-534G\rightarrow A\) \((n = 1)\), or in total seven patients carried a promoter mutation resulting in a mutation frequency in the whole group of 0.93% (7/750). We did not observe the
A variant that we previously identified in one Belgian patient but was shown not to affect in vitro APP transcription. We also did not observe the −118C→A mutation that we previously observed in one Dutch patient with onset age 50 years and increased APP transcriptional activity in vitro by 1.7-fold (Theuns et al., 2006). Either this mutation is extremely rare or is specific for the Dutch population. Population specificity of certain variants as a result of a different genetic background is supported by the absence from 435 Belgian control individuals of the two promoter variants −375G→C and −343A→C that we previously observed in two Dutch control individuals (Theuns et al., 2006). The fact that we only observed APP promoter mutations that are known to affect APP transcriptional activity and are located in the 200 bp fragment (−540 to −340) of the APP proximal promoter with the highest degree of interspecies conservation (>95%), supports an important role for cis-elements located in this region in APP transcriptional control.

The patients with a −369C→G or −534G→A mutation had a positive first-degree family history and in their families several members were reported, by family informants, to have dementia. The two patients carrying the −369C→G mutation are likely to be distantly related since they shared alleles at five neighbouring STR markers in a genomic region of 460–586 kb. The onset age in the three patients ranged from 61 to 78 years and the inheritance pattern in their families mimics that of families with autosomal dominant inheritance of APP coding or duplication mutations (Fig. 1). We currently have no in vivo data of APP expression or Aβ42 levels in brain since all patients are still alive. However, the profiles of the CSF biomarker panel (Aβ1–42, tau, P-tau181P) were confirmatory for the diagnosis of probable Alzheimer’s disease in two of the carriers (d1081 and d4904). Moreover, both carriers displayed low CSF Aβ42 levels (306 and 238 pg/ml, respectively). The most frequent promoter mutation, −479C→T was observed in four patients with onset ages between 62 and 79 years (mean 71.5 years). None of these patients had first-degree relatives with dementia. Haplotype sharing analysis was not supportive for a disease haplotype in the families of the two probands (Table 2). An APOE genotype data of the −369C→G mutation carriers suggested that the APOE ε4 allele lowered their onset age. Patient d1081, who is heterozygous for the ε4 allele, had an onset of disease at 78 years (Table 2). An APOE genotype effect was not observed for the −479C→T carriers (Table 2), suggesting that in these patients other modifying factors might be contributing to expression of disease. Further, previous linkage and association studies have indicated that genetic variability at the APP locus might contribute to the risk of developing late-onset Alzheimer’s disease (Wavrant-De Vrieze et al., 1999; Olson et al., 2001; Myers et al., 2002; Olson et al., 2002; Blacker et al., 2003). The −479C→T mutation, exhibiting a mild effect on APP expression and therefore resulting in later onset ages, could possibly underlie the observed linkage peaks that were most suggestive in APOE ε4-carriers. Although our data are consistent with these APP promoter mutations being associated with increased risk for the disease, we cannot fully exclude at this stage that their observation in control individuals points to a non-causal nature of these mutations or to reduced penetrance. One other caveat of this study is that we have not yet been able to test segregation of the −369C→G mutation in the families of the two probands (Fig. 1). Also, we have no in vivo data of APP transcript or protein levels in brain since all mutation carriers are still alive or died without autopsy.

In the group of patients (n = 204) with onset age ≤70 years we also examined the presence of APP coding or duplication mutations. We identified one, autopsy proven, patient (1/204 = 0.49%) with an APP missense mutation, namely the London APP Val717Ile mutation, one of the best documented mutations that has been observed so far in 29 families worldwide (http://www.molgen.ua.ac.be/ADMutations/). In the family we confirmed the presence of the mutation in one living patient and one at-risk individual. The mean onset age in the family was 57.7 ± 7.1 years (n = 3, range 50–64 years) and mean age at death of 69.5 ± 7.8 years (n = 2, range 64–75 years), which is 5–7 years later than reported on average for Val717Ile families (http://www.molgen.ua.ac.be/ADMutations/). This can be explained by the APOE ε2/ε3 genotype in the two patients that carried the mutation. The frequency of coding APP mutations in this Alzheimer’s disease group is comparable to previous reports (van Duijn et al., 1994; Zekanowski et al., 2003; Tanzi and Bertram, 2001). None of the patients carried an APP locus duplication; however, in our group there were only 39.7% patients that had a positive family history of dementia (Table 1), and so far duplications have only been reported in autosomal dominant patients (Rovelet-Lecrux et al., 2006; Skeiegers et al., 2006).

In conclusion, in this study we provided evidence that APP promoter mutations that affect APP transcriptional activity are apparently more frequently associated with increased
risk of Alzheimer’s disease than APP coding or duplication mutations. We also showed that the level of APP expression influences onset age in mutation carriers. Further, carriers of the same mutations had different onset ages pointing towards modifying factors. In patients carrying an APP promoter mutation increasing expression levels by near 2-fold, the disease was inherited comparable to families segregating an APP locus duplication or an APP missense mutation. Also, in these patients the APOE e4 allele might be one factor modulating onset age. This observation warrants testing for these mutations in a molecular DNA diagnostic setting. The −479C→T mutation was most frequently observed and potentially underlies previous observations of linkage and association with the APP locus in late-onset patients.

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