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ARTICLE

Mutation in the 3'untranslated region of *APP* as a genetic determinant of cerebral amyloid angiopathy

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Aβ-related cerebral amyloid angiopathy (CAA) is a major cause of primary non-traumatic brain hemorrhage. In families with an early onset of the disease, CAA can be due to amyloid precursor protein (*APP*) pathogenic variants or duplications. *APP* duplications lead to a ~1.5-fold increased *APP* expression, resulting in Aβ overproduction and deposition in the walls of leptomeningeal vessels. We hypothesized that rare variants in the 3'untranslated region (UTR) of *APP* might lead to *APP* overexpression in patients with CAA and no *APP* pathogenic variant or duplication. We performed direct sequencing of the whole *APP* 3'UTR in 90 patients with CAA and explored the functional consequences of one previously unreported variant. We identified three sequence variants in four patients, of which a two-base pair deletion (c.*331_*332del) was previously unannotated and absent from 175 controls of same ethnicity. This latter variant was associated with increased *APP* expression *in vivo* and *in vitro*. Bioinformatics and functional assays showed that the *APP* c.*331_*332del variant affected *APP* messenger RNA (mRNA) structure and binding of two microRNAs (miR-582-3p and miR-892b), providing a mechanism for the observed effects on *APP* expression. These results identify *APP* 3'UTR sequence variants as genetic determinants of Aβ-CAA.
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INTRODUCTION

Cerebral amyloid angiopathy (CAA) is characterized by amyloid deposits in the wall of cortical and leptomeningeal blood vessels, resulting in leukoencephalopathy with intracerebral hemorrhages (ICH). In postmortem studies, moderate to severe CAA affects 2.3% of the population aged between 65 and 74 years, and the prevalence increases to 8% among individuals aged 75–84 years, and 12.1% after 85 years of age[1] (for review see Biffi and Greenberg[2]). The most frequent components of the vascular deposits are the Aβ peptides (Aβ-CAA). These short (37–43 amino acids in length) Aβ peptides are generated from the proteolytic processing of the amyloid precursor protein (APP). Potential causes of Aβ deposition in vessels include increased production or reduced clearance mechanisms. The aggregation of Aβ peptides in the extracellular space is also a key pathological event in Alzheimer Disease (AD).

Most cases of Aβ-CAA are believed to occur sporadically. In addition to age, the ε4 allele of the *APOE* gene has been shown to be a common risk factor for CAA ($OR_{APOE\ \varepsilon4+} = 2.7$ [2.3–3.1]),[3] whereas studies suggesting a role of the *APOE* ε2 allele as a risk factor for CAA were inconsistently replicated. It has also been suggested that *APOE* ε2 could be a risk factor for ICH in vessels with amyloid charge.[4] More rarely, early-onset Aβ-CAA can be inherited as an autosomal dominant trait. Some cases are due to missense pathogenic variants in exons 16 and 17 of the *APP* gene, which encode the Aβ peptide and flanking amino acids. These variants (ie, Dutch, Italian, Arctic, Iowa, Flemish, and Piedmont) lead to increased production of aggregation-prone Aβ peptides. Interestingly, *APP* gene duplications cause early-onset autosomal dominant AD with Aβ-CAA.[5] Duplications of the *APP* gene result in a ~1.5- fold increase of APP expression, which is believed to be the direct cause of Aβ overproduction and EOAD with Aβ-CAA.[6] Patients with Down syndrome, who also carry three copies of the *APP* gene, have enhanced APP expression and are prone to develop AD with Aβ-CAA.[7] Increased *APP* gene expression is therefore a strong determinant for Aβ-CAA.

Recently, variants located in the 3' untranslated region (3'UTR) of *APP* were identified in a subset of patients with AD,[8] some of which

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Table 1 Patients with cerebral amyloid angiopathy (CAA)

Diagnostic classification[a]	*N*	*Mean age at onset*[b] *(min–max)*	*Mean age at last evaluation (min–max)*	*Sex ratio M/F*	*Percentage of cases with positive family history*[c]
Definite CAA	3	54.5 (54–55)	69 (63–75)	2/1	33.3%
Probable CAA with supporting pathology	2	63 (61–65)	63.5 (61–66)	1/1	0
Probable CAA	82	61.0 (31–82)	65.2 (40–90)	55/27	42.7%
Possible CAA	3	67 (53–77)	72 (63–77)	3/0	33.3%
Total	90	61.1 (31–82)	65.5 (40–90)	61/29	41.1%

[a]According to modified Boston criteria (Linn *et al*,[13]) with the exception of the criterion of age for possible and probable CAA.
[b]Any neurological event, for example, first intracerebral hemorrhage, ischemic stroke, onset of cognitive decline, seizure.
[c]Among first-degree relatives, history of stroke and/or cognitive decline (family information was not available for 12 patients).

affected microRNA (miRNA) binding and APP expression *in vitro*.[9] The short, regulatory miRNAs play an important role in posttranscriptional gene expression regulation (reviewed in Filipowicz *et al*[10]), whereas their involvement in neurodegenerative disorders is increasingly appreciated (reviewed in Hebert and De Strooper[11]). Interestingly, the *APP* 3'UTR is highly conserved across species,[12] strengthening the biological importance of this non-protein-coding region.

In this study, we aimed to determine whether sequence variants in the 3'UTR of *APP* could contribute to genetically unexplained cases of Aβ-CAA. To this end, we sequenced the complete 3'UTR of *APP* (~1200 bp) in 90 patients with CAA. We identified three *APP* 3'UTR sequence variants in four patients, including a previously unreported one (c.*331_*332del). This latter variant was associated with increased APP expression *in vivo* and *in vitro*. We provide evidence that the c.*331_*332del variant could affect APP mRNA structure and miRNA binding, providing a potential mechanism for increased APP expression. Together, these results reveal a previously unrecognized role of *APP* 3'UTR variants in Aβ-CAA, and further highlight the potential importance of miRNAs in the regulation of APP expression and neurological disorders.

MATERIALS AND METHODS

Patients

Blood samples from patients with CAA referred to two centers (CNR-MAJ, Rouen and Department of Genetics, Lariboisière Hospital, Paris, France) for *APP* sequencing and search for *APP* duplication were included in a national multicentric study. Diagnosis was performed according to the revised Boston diagnostic criteria[13] and ascertained by the two expert centers.

Patients fulfilling the criteria of definite CAA, probable CAA with supporting pathology, probable CAA, and possible CAA were included if they did not exhibit *APP* duplication (previously checked by QMPSF[5]) or *APP* pathogenic variant (previously checked by sequencing of exons 16 and 17). The criterion of age (Boston criteria require an age $\geq$55 years for the diagnosis of probable CAA) was not taken into account as we aimed at including patients with early as well as later onset of CAA. The study sample consisted of 90 patients (Table 1). First neurological event, age at onset, age at last clinical evaluation, and first-degree family history was extracted from medical records. All patients gave informed, written consent for genetic analyses. This protocol was approved by our ethics committees.

APP 3'UTR sequencing and expression analysis

DNA and RNA were extracted from whole blood. For Sanger sequencing, the whole *APP* 3'UTR was PCR-amplified using the following three sets of specific primers: A1 F-5′-TGTCCAAGATGCAGCAGAAC-3′ and R-5′-CTGAACT CCCACGTTCACAT-3′; A2 F-5′-CATAGCCCCTTAGCCAGTTG-3′ and R-5′-AATTGAAGACCAGCAGAGCA-3′; A3 F-5′-CCACGTATCTTTGGGTC TTTG-3′ and R-5′-AAGACACAACAGGTGTGGGTA-3′. PCR products were sequenced and analyzed on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Grand Island, NY, USA). Nomenclatures refer to NM_000484.3.

The new variant has been submitted to the Leiden Open Variation Database (LOVD, http://databases.lovd.nl/shared/variants/0000046545#01472).

RT-QMPSF was performed in triplicate experiments as previously described.[6] Briefly, RNA was reverse-transcribed into cDNA using the Verso cDNA kit (Fisher Scientific, Illkirch, France), and the single-stranded cDNA was PCR-amplified in a single reaction using two pairs of primers spanning exons 12/13 and 16/18 of *APP*, and three pairs of primers spanning three reference genes: *SF3A1*, *EIF4A2*, and *TOP1*. Sense primers were 6-FAM-labeled. RT-PCR were analyzed using the Genescan 3.7 Software (Applied Biosystems) after electrophoretic separation on a ABI Prism 3100 DNA sequencer (Applied Biosystems). Relative *APP* mRNA levels from the patient carrying the c.*331_*332del and his mother were normalized using the same sample of six healthy individuals constituting the normalization sample described in the study by Pottier *et al*.[6] Normalized results were then compared with the previously published series composed of 58 control individuals, 21 patients with Down syndrome, and 9 patients with an *APP* duplication.[6] Three *APP* duplication carriers were added to the series using the same procedures.

Controls

We compared results from Sanger sequencing of the *APP* 3'UTR with previously published data,[8] the exome variant server, the 1000 Genomes project, and dbSNP. In addition, we performed Sanger sequencing of the whole *APP* 3'UTR of 175 supplemental controls of the same ethnic origin (Sub-Saharan Africa) as the patient carrying the c.*331_*332del variant.[14]

In silico analyses of *APP* 3'UTR variants

Secondary structure analysis of human *APP* (*hAPP*) wild type (WT) or carrying the c.*331_*332del (named hereafter ΔTA) mRNA was performed using the CYCLOFOLD algorithm.[15] Candidate miRNAs were selected based on the PITA algorithm,[16] specifically designed to identify miRNA:mRNA interactions based on site accessibility.

Cell culture

Human HeLa cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum. One day before transfection, HeLa cells were plated at a 20% confluence in 6-well plates. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cellular assays

The full-length *hAPP* 3′UTR luciferase construct was described previously.[17] Mutagenesis was performed by TOPgene technologies (Montreal, Quebec, Canada) and validated by sequencing. Cells were transfected with or without 5 nM pre-miRs (Applied Biosystems), 2.5 ng/cm^2 pRL control vector, and 50 ng/cm^2 pGL3_HSV TK_3′UTR *hAPP* WT or ΔTA plasmids. Twenty-four hours post transfection, cells were lysed, and luciferase activity was measured according to the manufacturer's instructions (Promega, Madison, WI, USA).

RNA-binding protein immunoprecipitation (RIP)

The Ago2-RIP protocol was performed as described previously.[18] Snap-frozen human cortical brain tissue[19] was used as the material (control individuals, $n=2$, average age of 74 years old, average RIN values of 6.8). We used the Ago2 (Cell Signaling, Danvers, MA, USA; cat. #C34C6) and normal rabbit immunoglobulin (Cell Signaling cat. #2729) antibodies for immunoprecipitation. Immunoprecipitated RNAs (miRNA and mRNA) were subjected to quantitative real-time PCR, as before.[18] Oligonucleotides for *APP* are: F-5′-GTGTGCCCCATTCTTTTACG-3′ and R-5′-GGAAGTTTAACAGGATCT CGGG-3′, and for *NA4R1* are: F-5′-GCCTAGCACTGCCAAATT-3′ and R-5′-TC TGCCCACTTTCGGATAAC-3′.

RESULTS

We sequenced the 3'UTR of *APP* in 90 patients with definite ($n=3$), probable with supporting pathology ($n=2$), probable ($n=82$), and possible ($n=3$) CAA. Demographical characteristics are presented in Table 1. The first neurological event was acute symptomatic intracerebral lobar or subarachnoid hemorrhage in 46 patients, cognitive decline in 27 patients, ischemic stroke before the diagnosis of CAA in 7 patients, seizures not related to stroke in 6 patients, and cephalalgia not related to stroke in 2 patients. The diagnosis was fortuitous for one patient, and the presenting symptom was unknown in another one. The mean age at onset was 61.1 years (range 31–82).

We identified three sequence variants in four patients (Table 2). One variant, a two-base pair deletion, c.*331_*332del, was previously unreported. The other two were rare single nucleotide variants: c.*18C>T (rs201729239, reported once among 6503 individuals in the exome variant server, and once in the CS Agilent dataset, ss491814511) and c.*372A>G (allele frequency of 0.2% in the 1000 Genome project). We note that, in the exome variant server, only the first 50 bp after the stop codon were covered. Bettens *et al*[8] previously reported the results of Sanger sequencing of *APP* 3'UTR in 358 AD patients and 462 controls. Neither the c.*331_*332del nor the c.*18C>T variants were reported in the latter study. Conversely, the c.*372A>G variant was retrieved in both groups (0.3% frequency in AD group and 0.47% in controls). Taken together, this suggests that the c.*372A>G variant is a rare polymorphism.

The patient carrying the c.*18C>T variant was diagnosed with probable CAA at the age of 58 years. His first neurological event was a right middle-cerebral artery ischemic stroke, treated by aspirin. One month later, he presented lobar right temporal and parietal hematoma. Cerebral MRI revealed numerous cortical and juxtacortical microbleeds in all cerebral territories, together with white matter hyperintensities and no bleeding in deep gray matter. Five years later, he presented a novel spontaneous right lobar hematoma. He had no family history of CAA. RNA was not available for expression analysis.

The patient carrying the c.*331_*332del variant was diagnosed with probable CAA based on multiple spontaneous lobar and subarachnoid hemorrhages with diffuse superficial siderosis, starting from the age of 39 years. His personal medical history was marked by spina bifida with ventriculoperitoneal shunting at birth, which was properly functioning since first placement and after last revision, performed at 12 years. At the age of 39, he presented spontaneous cortical parietal bilateral subarachnoid hemorrhages revealed by cephalalgia and delirium. Replacement of shunting was decided. One week later, cerebral MRI showed a right frontal hematoma, distant from the shunting trajectory. Moreover, during the following months and in the absence of any new medical event or shunt malfunction, he presented several spontaneous lobar hematomas: latest cerebral MRI showed, at the age of 41, right temporal, frontal, fronto-temporal and left frontal and frontotemporal lobar cortical hematomas, as well as numerous cortical microbleeds in

Table 2 Variants found after Sanger sequencing of 3'UTR of *APP* in 90 patients with CAA

			Controls				*Patients with CAA*					
Variant nomenclature	*dbSNP id*	*PhyloP score*	*Exome variant Server*	*1000 Genomes*	*Bettens et al.*[a]	*175 African controls (Sanger)*	*Count*	*AAO*	*APOE*	*Clinical summary*	*Family history*	*Interpretation*
c.*18C>T	rs201729239	4.24	EA:0/4300 AA:1/2203 MAF: 0.0077%	1/297 (CS Agilent, Europe, WES)	0%	0	1/90	58	23	58 years: Ischemic stroke, and then one lobar ICH 63: lobar ICH MRI: WMH, numerous cortical and juxtacortical microbleeds	Negative	Unknown effect
c.*331_*332del	Unreported	c.*331: 4.00 c.*332: 2.38	Not covered	Unreported	Unreported	0	1/90	39	34	Focal SAH, then recurrent lobar ICH, cortical and juxtacortical microbleeds, WMH	Negative	Rare functional variant
c.*372A>G	rs187940037	1.66	Not covered	4/1094 MAF = 0.2%	0.47%	0	2/90	73	34	Focal SAH, cortical and juxtacortical microbleeds, WMH	Negative	Probable rare polymorphism
								53	44	2 lobar ICH, cortical and juxtacortical microbleeds	Mother: ICH at age 83	

Abbreviations: AAO, age at onset; ICH, lobar intracerebral hemorrhage; MAF, minor allele frequency; SAH, subarachnoid hemorrhage.
[a]462 controls from Bettens *et al*,[8] frequency based on the actual number of successful sequences.

all cerebral territories, diffuse superficial siderosis (Figure 1), white matter hyperintensities, and no bleeding in deep gray matter. Taking these arguments together, the diagnosis of CAA was highly probable. He had no family history of CAA. His *APOE* genotype was 3–4. Neither *APP* pathogenic variant nor *APP* locus duplication was detected. Sequencing of *APP* 3'UTR revealed a previously unreported two-base pair deletion, c.*331_*332del (r.*331_*332del). The patient was from Sub–Saharan African origin. To make sure that this variant is not frequently encountered in this ethnic group, we screened the presence of this variant in 175 controls originating from the same region of Africa. None of them carried this variant. The patient's unaffected mother did not carry the c.*331_*332del variant. The patient's father's DNA was unavailable (sudden death at the age of 70 by unknown cause). Expression analyses by RT-QMPSF revealed a ~1.5-fold increased level of the *APP* mRNA transcript in the patient (mean ± SD: 1.47 ± 0.06), while the analysis of his mother's RNA revealed a normal level of the *APP* mRNA transcript (1.12 ± 0.06, Table 3).

As the c.*331_*332del variant was unreported from databases and absent in controls, and as expression analysis showed increased levels of *APP* transcripts in the patient affected by probable CAA, we focused on this variant for functional analyses.

The c.*331_*332del variant is located in a highly conserved region of the *APP* 3'UTR (Figure 2a). To evaluate the effects of c.

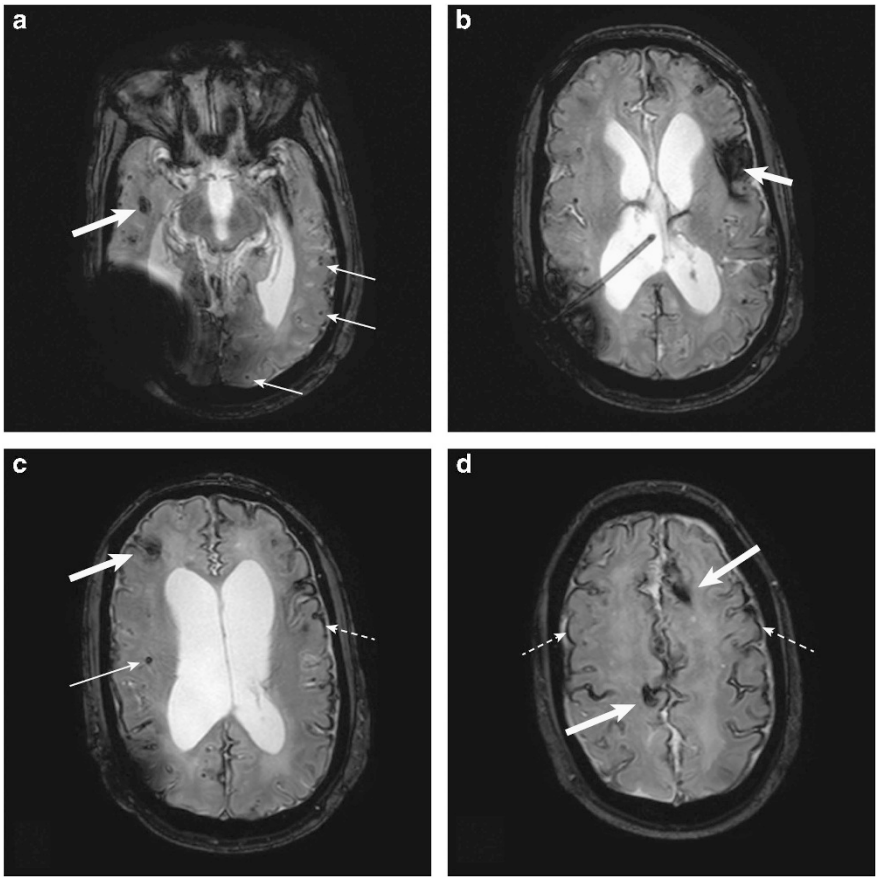

Figure 1 T2*-weighted cerebral MRI of the patient carrying the *APP* c.*331_*332del variant at the age of 41 years, showing cortical and subcortical microbleeds (**a** and **c**, solid arrows), lobar hematomas (**a**–**d** heavy arrows) and superficial siderosis (**c** and **d**, dotted arrows).

Table 3 APP mRNA expression in blood

	n	*Mean*	*SEM*[a]	*Median*	*Range*
Controls[b]	58	1.01	0.02	0.99	0.68–1.46
Down syndrome	21	1.46	0.07	1.49	0.80–2.05
APP duplication	12	1.40	0.10	1.39	0.79–1.89
Patient with c.*331_*332del	1			1.47 ± 0.06[c]	
Unaffected mother, not carrying the c.*331_332 del mutation	1			1.12 ± 0.06[c]	

Relative APP mRNA levels in 58 control individuals, 21 patients with Down syndrome, 12 patients with *APP* duplication[6], and the patient carrying the c.*331_*332del mutation in the *APP* 3'UTR. Each individual value corresponds to the average of three measures by RT-QMPSF.
[a]Standard error of mean among the 58 controls, the 21 patients with Down syndrome, and the 12 patients with *APP* duplication, respectively, as a reflect of inter-individual variation.
[b]Mean age ± SEM: 64.6 ± 1.16.
[c]Standard error of mean of the three analysis replicates of RT-QMPSF in the patient carrying the c.*331_*332del mutation and his unaffected mother, respectively, as a reflect of measure variability.

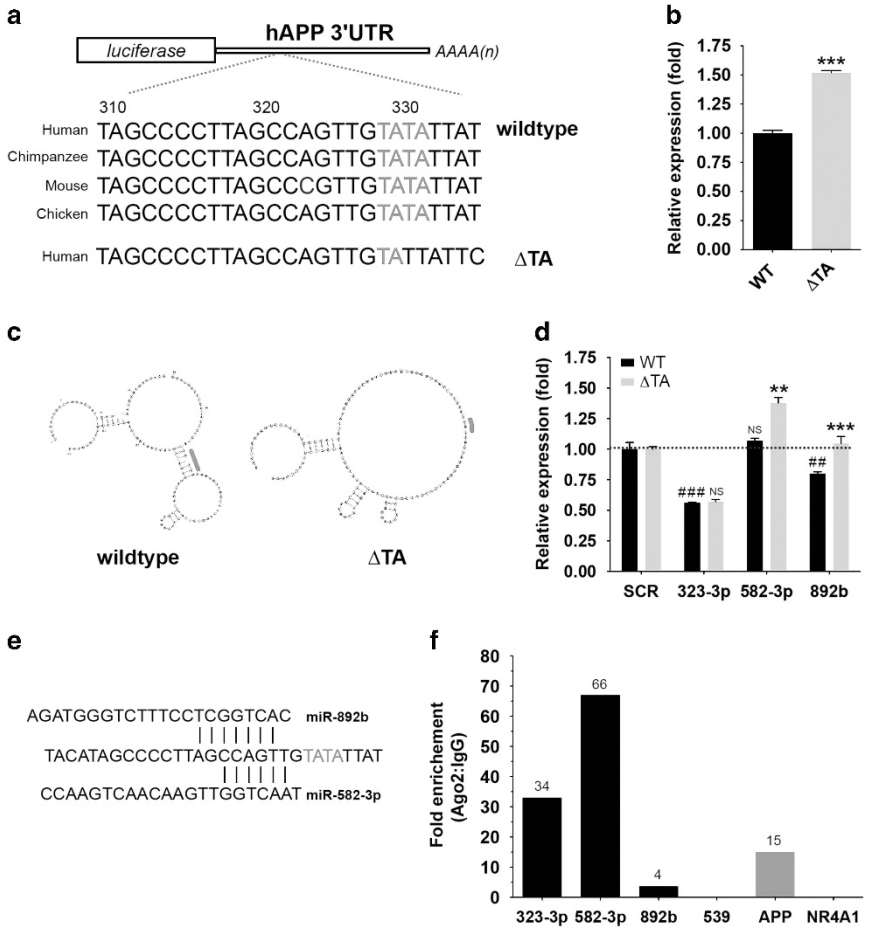

Figure 2 (**a**) Schematic representation (not to scale) of the hAPP luciferase reporter construct used in this study. TK, thymidine kinase promoter; AAAA(n), PolyA site. Note that nucleotides surrounding the TATA site (in light grey) are highly conserved (shown here are human, chimpanzee, mouse, and chicken sequences). The APP deletion (ΔTA) is depicted. Nucleotide positions in the hAPP 3'UTR are shown. (**b**) HeLa cells were co-transfected with hAPP WT or ΔTA constructs and Renilla (used as normalizing control). Relative expression (in fold) is shown after normalization ($n=3$ in triplicate). Statistical significance was assessed using an unpaired *t*-test (***$P<0.001$). (**c**) Secondary structure analysis of hAPP WT or ΔTA mRNA. Here are shown ~70 nucleotides flanking the TATA sequence (underlined in red). (**d**) HeLa cells were transfected with 5 nM miRNAs (see list) as well as hAPP WT or ΔTA constructs. Signals were normalized for transfection efficiency, and graph represents the luciferase signals compared with the scrambled control (SCR) ($n=3$ in triplicate). Statistical significance was assessed using ANOVA (**$P<0.01$; ***$P<0.001$ when compared with WT and $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ when compared with SCR). NS, Non significant. (**e**) miRNA:mRNA alignment analysis of miR-582-3p and miR-892b with the APP 3'UTR. Note that predicted miRNA seed sequences are flanking the TATA sequence at the 5' end. (**f**) Representative immunoprecipitation of Ago2-associated RNAs in the brain. Mature candidate miRNAs (miR-582-p and miR-892b) as well as APP mRNA are enriched in the functional ('loaded') miRNA/RISC complex. Both miRNAs and mRNAs were measured by qRT-PCR ($n=2$, measured in triplicate). MiR-323p was used as the positive control for the RIP experiments, while miR-539 and NR4A1 mRNA were used as negative controls. Fold enrichments were calculated as Ago2 RIP *versus* IgG RIP.

*331_*332del on APP expression *in vitro*, we cloned the full-length *hAPP* 3'UTR downstream of a luciferase reporter (Figure 2a).[9] The c.*331_*332del variant (hereafter, named ΔTA) was included by site-directed mutagenesis. When compared with the control (WT) sequence, the ΔTA variant caused a ~1.5-fold increase in luciferase (APP) expression once incorporated into human HeLa cells (Figure 2b), consistent with our patient data. Thus, the presence of the c.*331_*332del variant alone is sufficient to cause an increase in APP expression.

We next searched for potential mechanisms involved in ΔTA-induced APP expression upregulation. Bioinformatics analysis using the CYCLOFOLD algorithm[15] showed drastic changes in APP mRNA secondary structure in the presence of the ΔTA variant (Figure 2c). Alterations in mRNA secondary structure are known to influence miRNA binding and function. To test this hypothesis, we used the PITA algorithm,[16] specifically designed to identify miRNA:mRNA interactions based on site accessibility. This program identified miR-582-3p and miR-892b potential binding sites located near the ΔTA sequence (Figure 2d and e). To validate these predictions, we co-transfected candidate miRNAs with the APP luciferase reporters in HeLa cells. Upon co-transfection, the ΔTA variant increased luciferase (APP) expression when compared with the WT sequence (Figure 2d). Notably, miR-892b significantly downregulated *APP* expression, whereas the ΔTA variant blocked this effect. Interestingly, miR-582-3p caused an increase in *APP* expression only in the presence of the ΔTA variant. As a positive control for this experiment, we used the previously identified miR-323-3p, which targets another region in the APP 3'UTR[9] and is independent on mRNA secondary structure (not shown). As expected, miR-323-3p miRNA was not affected by the ΔTA variant. We finally performed Ago2 RIP experiments[18] on

human post-mortem tissue. These assays showed that mature (functional) miR-582-3p and miR-892b associate with APP mRNA under physiological conditions in the adult human brain (Figure 2f). Taken together, these results suggest that the loss of miRNA binding is responsible, at least in part, for the increased expression of APP in patients with the c.*331_*332del variant.

DISCUSSION

We report the first genetic screening of the 3'UTR of the *APP* gene in a case series of patients with CAA. Sequence variants of *APP* 3'UTR were found with a low frequency in our case series (4/90, 4.4%). Among the three variants identified, a previously unreported one, c.*331_*332del, was associated with increased *APP* expression *in vivo* in a patient with probable CAA. This increased *APP* expression could be attributed, at least in part, to the abnormal binding of two miRNAs (miR-582-3p and miR-892b) to the APP mRNA. These results also suggest that the two aforementioned miRNAs, which are expressed in human brain and co-immunoprecipitate with the active miRNA complex and APP mRNA, are involved in the regulation of *APP* expression. Importantly, the levels of *APP* transcripts observed in the patient carrying the variant were comparable with patients with *APP* duplications[6] and to our *in vitro* cellular assays. We suggest that this variant, resulting in a significant increase of *APP* expression, is a strong genetic determinant for CAA. The variant was not inherited from the unaffected mother, but the father's DNA was not available, which did not allow us to determine whether the variant occurred *de novo*.

Another variant, c.*18C>T, was reported with an extremely low frequency in control databases. Owing to the lack of material, we could not test *APP* expression. However, we cannot exclude that this variant is involved in the genetic determinism of probable CAA.

Previous studies have shown that APP is regulated at both transcriptional and posttranscriptional levels.[10,20] Association studies about sequence variants in *APP* proximal and distal promoter regions gave inconsistent results in the risk of AD.[20–23] Some of these studies showed an increase in *APP* expression *in vitro*, but did not provide *in vivo* validation.[20] *APP* promotor screening has not yet been performed in CAA, to our knowledge. We and others have also shown that *APP* expression can be regulated by several miRNAs, and it has been hypothesized that genetic variations in the 3′UTR of *APP* that either abolish existing miRNA-binding sites or create illegitimate miRNA-binding sites could significantly contribute to the risk for disease (reviewed in Delay *et al*[24] and Long *et al*[25]). Our previous studies reported that variants within the 3'UTR of *APP*, found in several patients with AD, can increase *APP* expression level *in vitro*, but these data could not be assessed *in vivo*.[9] Here, we provide the first evidence that a patient-specific sequence variant within the 3'UTR of APP, found in a patient with probable CAA, may influence *APP* transcript levels *in vivo* in humans.

Increased *APP* expression has been shown to cause CAA with AD.[5] Some of the patients from the present case series may be affected by both CAA and AD. On the contrary, as CAA is most of the time a probabilistic diagnosis (in absence of neuropathological data, in 85/90 patients here), we cannot exclude that some of our patients might eventually not be affected by definite CAA or might be affected by CAA not related to Aβ.

Of note, the patient carrying the c.*331_*332del variant had a personal history of spina bifida of unknown cause. ICHs were temporally and spatially distinct from surgery and material, respectively, and were associated with numerous cortical microbleeds, confirming that the patient had two distinct diseases. We hypothesize that this could be coincidental.

Although the mean age at onset was 61.1 years in our study, we included both young and older patients (range: 31–82 years). Three out of four variants were identified in patients with an age of onset before 60 years (2/2 if excluding the most likely polymorphic c.*372A>G variant). Previous studies focused on patients with AD, especially late-onset AD.[8] Increased expression of *APP* due to *APP* duplications has been shown to cause early-onset AD with CAA.[5] For these reasons, we propose that genetic screening of the 3'UTR of *APP* should be performed in patients with early-onset AD, early-onset CAA, or both, if no *APP* pathogenic variant or duplication was previously identified.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Greenberg SM, Vonsattel JP: Diagnosis of cerebral amyloid angiopathy. Sensitivity and specificity of cortical biopsy. *Stroke* 1997; **28**: 1418–1422.
2 Biffi A, Greenberg SM: Cerebral amyloid angiopathy: a systematic review. *J Clin Neurol* 2011; **7**: 1–9.
3 Rannikmae K, Samarasekera N, Martinez-Gonzalez NA, Al-Shahi Salman R, Sudlow CL: Genetics of cerebral amyloid angiopathy: systematic review and meta-analysis. *J Neurol Neurosurg Psychiatry* 2013; **84**: 901–908.
4 McCarron MO, Nicoll JA: Apolipoprotein E genotype and cerebral amyloid angiopathy-related hemorrhage. *Ann NY Acad Sci* 2000; **903**: 176–179.
5 Rovelet-Lecrux A, Hannequin D, Raux G *et al*: APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat Genet* 2006; **38**: 24–26.
6 Pottier C, Wallon D, Lecrux AR *et al*: Amyloid-beta protein precursor gene expression in alzheimer's disease and other conditions. *J Alzheimers Dis* 2012; **28**: 561–566.
7 Donahue JE, Khurana JS, Adelman LS: Intracerebral hemorrhage in two patients with Down's syndrome and cerebral amyloid angiopathy. *Acta Neuropathol* 1998; **95**: 213–216.
8 Bettens K, Brouwers N, Engelborghs S *et al*: APP and BACE1 miRNA genetic variability has no major role in risk for Alzheimer disease. *Hum Mutat* 2009; **30**: 1207–1213.
9 Delay C, Calon F, Mathews P, Hebert SS: Alzheimer-specific variants in the 3'UTR of Amyloid precursor protein affect microRNA function. *Mol Neurodegener* 2011; **6**: 70.
10 Filipowicz W, Bhattacharyya SN, Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; **9**: 102–114.
11 Hebert SS, De Strooper B: Alterations of the microRNA network cause neurodegenerative disease. *Trends Neurosci* 2009; **32**: 199–206.
12 Hebert SS, Nelson PT: Studying microRNAs in the brain: technical lessons learned from the first ten years. *Exp Neurol* 2012; **235**: 397–401.
13 Linn J, Halpin A, Demaerel P *et al*: Prevalence of superficial siderosis in patients with cerebral amyloid angiopathy. *Neurology* 2010; **74**: 1346–1350.
14 Guerchet M, Mbelesso P, Ndamba-Bandzouzi B *et al*: Epidemiology of dementia in Central Africa (EPIDEMCA): Protocol for a multicentre population-based study in rural and urban areas of the Central African Republic and the Republic of Congo. *Springerplus* 2014; **3**: 338.
15 Bindewald E, Kluth T, Shapiro BA: CyloFold: secondary structure prediction including pseudoknots. *Nucleic Acids Res* 2010; **38**: W368–W372.
16 Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E: The role of site accessibility in microRNA target recognition. *Nat Genet* 2007; **39**: 1278–1284.
17 Hebert SS, Horre K, Nicolai L *et al*: MicroRNA regulation of Alzheimer's Amyloid precursor protein expression. *Neurobiol Dis* 2009; **33**: 422–428.
18 Dorval V, Mandemakers W, Jolivette F *et al*: Gene and MicroRNA transcriptome analysis of Parkinson's related LRRK2 mouse models. *PloS One* 2014; **9**: e85510.
19 Hebert SS, Wang WX, Zhu Q, Nelson PT: A study of small RNAs from cerebral neocortex of pathology-verified Alzheimer's disease, dementia with lewy bodies, hippocampal sclerosis, frontotemporal lobar dementia, and non-demented human controls. *J Alzheimers Dis* 2013; **35**: 335–348.
20 Theuns J, Brouwers N, Engelborghs S *et al*: Promoter mutations that increase amyloid precursor-protein expression are associated with Alzheimer disease. *Am J Hum Genet* 2006; **78**: 936–946.
21 Lahiri DK, Ge YW, Maloney B, Wavrant-De Vrieze F, Hardy J: Characterization of two APP gene promoter polymorphisms that appear to influence risk of late-onset Alzheimer's disease. *Neurobiol Aging* 2005; **26**: 1329–1341.

22 Guyant-Marechal L, Rovelet-Lecrux A, Goumidi L *et al*: Variations in the APP gene promoter region and risk of Alzheimer disease. *Neurology* 2007; **68**: 684–687.
23 Nowotny P, Simcock X, Bertelsen S *et al*: Association studies testing for risk for late-onset Alzheimer's disease with common variants in the beta-amyloid precursor protein (APP). *Am J Med Genet B Neuropsychiatr Genet* 2007; **144B**: 469–474.
24 Delay C, Mandemakers W, Hebert SS: MicroRNAs in Alzheimer's disease. *Neurobiol Dis* 2012; **46**: 285–290.
25 Long JM, Ray B, Lahiri DK: MicroRNA-153 physiologically inhibits expression of amyloid-beta precursor protein in cultured human fetal brain cells and is dysregulated in a subset of Alzheimer disease patients. *J Biol Chem* 2012; **287**: 31298–31310.