

ORIGINAL ARTICLE

Evidence for induction of the ornithine transcarbamylase expression in Alzheimer's disease

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To more rapidly identify candidate genes located within chromosomal regions of interest defined by genome scan studies in Alzheimer's disease (AD), we have developed a customized microarray containing all the ORFs ($n=2741$) located within nine of these regions. Levels of gene expression were assessed in total RNA from brain tissue of 12 controls and 12 AD patients. Of all genes showing differential expression, we focused on the ornithine transcarbamylase (OTC) gene on Xp21.1., a key enzyme of the urea cycle which we found to be expressed in AD brains but not in controls, as confirmed by RT-PCR. We also detected mRNA expression of all the other urea cycle enzymes in AD brains. Immunocytochemistry experiments revealed that the OTC expression was strictly restricted to vascular endothelial cells in brain. Furthermore, OTC activity was 880% increased in the CSF of probable AD cases compared with controls. We analysed the association of the OTC -389 G/A and -241 A/G promoter polymorphisms with the risk of developing AD. We observed that rare haplotypes may be associated with the risk of AD through a possible modulation of the methylation of the OTC promoter. In conclusion, our results suggest the involvement of a new pathway in AD brains involving the urea cycle.

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Introduction

The molecular genetic basis of the most common dementia in the elderly, Alzheimer's disease (AD) remains unclear. Hereditary early-onset forms of AD account for less than 5% of the total number of cases and these have been linked to mutations in three different genes: the amyloid precursor protein (*APP*) gene on chromosome 21, the presenilin 1 (*PS1*) gene on chromosome 14 and the presenilin 2 (*PS2*) gene on chromosome 1.¹ The aetiology of sporadic late-onset forms of AD is far more complex with the possible involvement of, and interaction between, environmental factors and various genes. Apolipoprotein E (*APOE*), especially the *APOE* $\epsilon 4$ allele, has been established as a strong susceptibility marker that accounts for approximately 20% of the genetic risk in late-onset AD. Genome-wide linkage or linkage dis-

equilibrium (LD) studies on late-onset AD forms have provided evidence for the existence of multiple putative genes on several chromosomal regions.² However, these regions are often broad and may contain several candidate genes.³

Thus, to speed up the selection of candidate genes within these regions of interest, we combined the genetic map information with gene expression profiling data. This strategy results from two major observations: (i) the expression of numerous genes is modified during AD aetiology,^{4–8} (ii) polymorphisms within promoters of the *APOE*, *PS1*, *PS2* and *APP* genes have been associated with the occurrence of AD.^{9–12}

Consequently, we assumed that genes located in one of the loci of interest defined by previous genome scans and exhibiting a differential expression between patients and controls, could constitute potential candidate genes for AD. We applied our strategy to nine different chromosomal regions previously identified by genome scan studies¹³ and selected a candidate gene expressed in cases but not in controls, the ornithine transcarbamylase (OTC) located on Xp21.1. We then tested the plausibility for this gene to be associated in AD physiopathology.

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Materials and methods

Microarray analyses

Brains used in transcriptomic experiments were obtained at autopsy from 114 patients with early- and late-onset sporadic AD accessioned from the Greater Manchester region of United Kingdom during years 1986–2001 (mean age at death = 73.1 ± 9.1 years old; mean age at onset = 65.9 ± 10.3 years old; 51% male). All patients were of Caucasian ethnic origin. Pathological diagnoses were made in accordance with CERAD Neuropathological Criteria for AD.¹⁴ All patients were at Braak stages 5 or 6 at time of death. Control brains were obtained from an initial set of 167 brains recruited from routine autopsies carried out at the Hospices Civils de Strasbourg (France). Recruitment was designed to exclude cases of dementia (Individuals were not recruited from medical institutions where the majority of patients presented with dementia, but from a general hospital). Most cases were admitted less than 48 h before death via emergency services and were living at home prior to their admission. Cases referred to autopsy for neurological pathologies were excluded. The neuropathological diagnosis for AD followed CERAD Neuropathological criteria. In addition, Braak stages for neurofibrillary tangles¹⁵ were assessed in the whole series. Again, all control subjects were Caucasian.

Total RNA was extracted from frozen frontal cortex brain tissue from all 114 AD and 167 control samples using phenol/chloroform protocol (TRIzol reagent, Invitrogen, Carlsbad, CA, USA). The quality of total RNA was assessed using Agilent 2100 bioanalyser and the ratio of ribosomal RNA 28S/18S systematically estimated using the Agilent 2100 bioanalyser bio-sizing software. A total of 12 AD cases and 12 controls were finally selected from the initial samples according to criteria: (i) a ratio of ribosomal RNA 28S/18S greater than or equal to 1.0; (ii) a Braak stage below 2 for the control samples. The main characteristics of the samples are shown in Supplementary Table S1 (Supplementary material).

Specific oligonucleotides for 2741 open reading frames (ORFs) located within the regions of interest defined by genome scan studies were designed using the OLIGOMER software (Mediagen). The main criteria of selection were (i) a length of 60 oligonucleotides; (ii) the hybridization temperature (between 65 and 75 °C); (iii) the specificity of the oligonucleotide sequence; (iv) inability to form a secondary structure at the hybridization temperature; (v) an oligonucleotide sequence close to the 3'-UTR end of the selected ORF (the oligonucleotide sequences are available at http://www.pasteur-lille.fr/fr/public_biopuces/Alzheimer). After synthesis of the oligonucleotides, these were systematically purified in order to obtain a population homogeneous in length (Sigma, St Louis, MO, USA). All the oligonucleotides were functionalized with a C₆H₁₂NH₂ arm at their 5' end.

We compared the genetic expression of each AD case compared with a pool of the control samples in order to decrease potential inter-individual variability in the control population. CRNAs—representative of the initial mRNA population from 10 µg of total RNA—were produced by amplification and labelled by Cy5 or Cy3 fluorophores using the Agilent Fluorescent Linear Amplification Kit as described by the supplier. We followed a dye-swap strategy, with each AD sample being analysed on two independent microarrays on which the same sample was labelled either by Cy3 or Cy5 fluorophores. For hybridization, 4 µl of cRNA from each AD case was mixed with 4 µl of cRNA from the control pool. This mix was then dissolved in 22 µl of hybridization buffer (Supplier) to obtain a final concentration of 40% formamide, 2.5 × Denhardt's, 0.5% sodium dodecyl sulphate (SDS) and 4 × saline sodium citrate (SSC). After incubating at 95 °C for 5 min, the mix was applied to the slides under a cover slip. The slides were then placed in a hybridization chamber (Corning, New York, NY, USA), and 30 µl of hybridization buffer was added to the chamber before sealing. The sealed chambers were incubated for 14–16 h in a water bath at 42 °C. The slides were then washed twice in SSC 2 × and SDS 0.1% for 5 min at 42 °C, once for 1 min in SSC 0.2 × at room temperature and then once for 1 min in SSC 0.1 × at room temperature. Finally, the slides were dried by centrifugation at 1000 r.p.m. for 5 min at room temperature. After hybridization, arrays were scanned using an Affymetrix 418 scanner and images were processed using ImaGene 6.0 (Biodiscovery) software. Raw data (available at http://www.pasteur-lille.fr/fr/public_biopuces/Alzheimer) were then analysed using the LIMMA library (Linear Models for Microarray Data)¹⁶ running under the statistical language R v2.0.1.¹⁷ A normalization protocol, consisting of a within-array print-tip less normalization to correct for dye and special effects,¹⁸ was applied on the background subtracted median intensities of the unflagged spots. After normalization, identification of statistically significant regulation was performed using moderated *t*-statistic with empirical Bayes shrinkage of the standard errors.¹⁹

Design strategy

The main goal of this study was to research for new genetic determinants of AD based on a biological convergence approach. An option would have been to use a pan-genomic commercial microarray such as the U133A affymetrix one. However, according to our goal, a restricted number of chromosomal regions defined by genome scan studies were of interest. These regions were known to contain one or several candidate genes of AD. We first annotated 5849 ORFs but 3101 were excluded following a systematic bioinformatic analysis.¹³ We demonstrated that these 3101 ORFs—even if referenced in the international databases—did not have a biological relevance. We noticed that a non-negligible part of them were nonetheless present on a commercial pan-genomic

microarray such as the U133A affymetrix one. Furthermore, we compared the listing of the 2741 genes we finally selected, with the genes available on the U133A affymetrix microarray. We noticed that only 48% of them were in common. In conclusion, this microarray allowed us for focusing on a restricted number of real genes (2748). This selection was nonetheless exhaustive to address our questioning and more precise than the information available on the U133A commercial microarray.

One of the main difficulties in differential expression analyses is to determine whether a variation is due to the pathological process or is a consequence of an inter-individual variability or technical bias. From these considerations, we first followed a dye-swap strategy to minimize the variation of hybridization between the pathological and control samples and to systematically perform duplicate experiments. According to this technology, we had to define the most pertinent number of experiments. Three main options were obvious at this stage: (i) each AD sample was compared with each control sample (288 microarrays). This option was nevertheless not realistic because of the limited amount of total RNA available from the control brains and its prohibitive cost; (ii) each AD sample was compared with a pool of all the control samples (48 microarrays); (iii) a pool of the AD samples was compared with a control pool (2 microarrays).

However, we postulated that the measure of an inter-individual variability in the controls was not relevant for our main objective—that is characterization of genetic risk factors for AD—and may even add some noise background. By opposition, the knowledge of this inter-individual variability in the brain of AD cases seemed to be more important to address our questioning. Furthermore, we determined that a statistical analysis (moderated *t*-statistic with empirical Bayes shrinkage of the standard errors) was well adapted to take into account the experiments altogether but also to evaluate the homogeneity of the difference in expression between the different AD cases. That is why accordingly to all these considerations, we decided to analyse each AD samples versus a pool of all the control samples.

RT-PCR

Reverse-transcription was performed from 500 ng of total RNA extracted from frontal cortex of 11 AD cases and 9 controls initially used for the microarray experiments. Specific amplifications of mRNAs from the carbamoyl-phosphate synthetase 1, *OTC*, argininosuccinate synthetase 1 (*ASS1*), argininosuccinate lyase (*ASL*) and arginase 1 genes were obtained using oligonucleotide sets described in Supplementary Table S2 (Supplementary material). Primers were designed within different exons to avoid potential contamination by amplification of genomic DNA. Control experiments were performed by omitting RNA sample. The PCR products were analysed on agarose gels (3%).

Immunohistochemistry experiments

Brains used for immunohistochemistry experiments were independent of those used in transcriptomic experiments and were obtained at autopsy at the University hospital of Lille from 12 Caucasian patients suffering from AD (seven men and five women, ranging from 57 to 95 years; mean age 75.3 years) and 4 Caucasian controls (patients devoid of any neurological disease, in whom the neuropathological study did not show any Alzheimer pathology, mean age 69.5 years). In all patients, the Alzheimer pathology was confirmed by immunohistochemistry and western blot analysis of Tau, A β and α -synuclein.²⁰ Brain samples were fixed in formalin for light microscopy examination. Paraffin sections from the anterior frontal cortex (BA 10) were processed in a Benchmark-XT automate (Ventana, Tucson, AZ, USA).

Anti-peptide polyclonal antibodies (pAbs) against a 20-amino acid polypeptide, specific of the human OTC protein (MKTAKVAASDWTFLHCLPRK), were developed from a standard protocol (immunization of 3 months, Proteogenix SA, France). The anti-OTC antibody and the pre-immune rabbit serum (both diluted 1/500) were applied after heating, and revealed by a standard immunoperoxidase technique. Positive controls were paraffin sections of formalin-fixed liver. Negative controls were brain sections from the Alzheimer patients and controls, processed with a pre-immune rabbit serum.

OTC activity in CSF

Cerebrospinal fluid (CSF) was obtained by lumbar puncture in 15 French probable AD and 11 controls without central nervous neurodegenerative disorders (AD cases age = 66.3 ± 15.6 years, 55% male; controls age = 47.0 ± 20.9 years, 66% male).

The OTC activity was quantified by the measurement of citrulline production in 30 min in the presence of its substrates in excess (carbamylphosphate and ornithine, Supplementary Figure S1). Citrulline production was next colorimetrically measured by a diacetylmonoxime–thiosemicarbazide reaction as described²¹ and according to a slightly modified protocol.²² For each sample, duplicates (with or without substrates) were performed in order to differentiate the amount of citrulline naturally present in the samples from the citrulline production due to OTC activity.

Briefly, CSF samples (50 μ l) were added either to a solution (140 μ l) containing ornithine (5 mM), lithium carbamylphosphate (15 mM) and triethanolamine (270 mM) or to distilled water (140 μ l). Following incubation (30 min) at 37 °C, the enzymatic reaction was stopped by addition of 3:1 (v/v) phosphoric acid/sulphuric acid (50 μ l). The colorimetric reaction was finally realized following addition of 2,3 butanedione (3%; 10 μ l) at 95 °C in the dark (15 min). Analyses were carried out on a microplaque reader (Elx800-Biotek) at 490 nm wavelength.

Two concentration scales were systematically carried out in each 96-well plate, one including increasing quantities (0–150 nmol per 50 μ l) of commercial citrulline, the other including increasing quantities (0 to 3.10–3 U per 50 μ l) of commercial OTC. Duplicates were performed in the absence or in the presence of substrates, as previously described. In order to assess the specificity of our experiment, we also pre-incubated the commercial OTC enzyme or a sample with a saturating quantity of the pAb described in the immunohistochemistry section (Supplementary Figure S2). Optical density (OD) values were finally deferred on the corresponding citrulline concentration scale in order to estimate the citrulline amount (nM) in each well. For each sample, the citrulline amount in absence of substrates was withdrawn from the quantity of nmol of citrulline measured in the presence of substrates. The resulting quantity indicated the citrulline level, produced by OTC in 30 min. We chose to express the OTC activity in nmol of *de novo* produced citrulline per 30 min per 50 μ l CSF.

Genotyping

Genotyping of eight single nucleotide polymorphisms (SNPs) was determined by enzymatic digestion following PCR amplification (Supplementary Table S3). A total of 50% of the genotypes were randomly performed twice and no discrepancy was observed.

AD case-control study

The French AD and control samples were Caucasian (AD cases $n = 583$, age = 72.4 ± 7.2 years, age at onset = 69.5 ± 7.4 years, 39.5% male; controls $n = 639$, age = 72.5 ± 7.9 years, 36% male). An early age at onset was defined as ≤ 65 years. A diagnosis of probable AD was established according to DSM-III-R and NINCDS-ADRDA criteria. Caucasian controls were recruited and defined as subjects without DMS-III-R dementia criteria, with integrity of cognitive function and with a mental state score ≥ 25 . Presence of family history of dementia was an exclusion criterion. Controls were recruited in retirement homes or from electoral rolls (altruistic volunteers). Each individual or next of kin gave informed consent.

Methylation status at position –389 and –241

In order to determine the methylation status of the cytosines of the CpG motifs at position –389 and –241 within the human *OTC* promoter, treatment of genomic DNA by bisulphite was performed using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA, USA). Briefly, 1 μ g of genomic DNA extracted from peripheral blood lymphocytes was treated with sodium bisulphite and hydroquinone and incubated at 50 °C for 16 h. Following this treatment, unmethylated cytosines were converted to uracil and methylated cytosines remained unchanged. After purification, the bisulphite-modified DNA was immediately used for PCR or stored at –70 °C.

Bisulphite-modified DNA (20 ng) was used as template for PCR in order to determine the methylation status of the CpG/A motif at position –389 using the primer set: 5'-ATAAATGTGAAGTTGTAGAT-5' and 5'-TAATTACCTATTAATTCTAAC-3'. The amplification product was next re-amplified using the primer set: 5'-GAATAGGTTGTTAGGGGAAG-3' and 5'-ATAAATGTGAAGTTGTAGAT-3'. A similar protocol was performed for the determination of the methylation status of the CpA/G motif at position –241 using the sets of primers: 5'-TGGGTTTATTGTAATTTTTGTTTTTT-3' and 5'-CTAACCAACATAA TAAATCCCCCATC-3'. The PCR fragments from individuals bearing either the GG or AA genotypes (four individuals by genotype) for both *OTC* promoter SNPs were cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) and at least five clones with appropriate sized inserts were sequenced for each individual.

Statistical analysis

The SAS software, release 8.0, was used (SAS institute, Cary, NC, USA). Univariate analyses were performed with Pearson's χ^2 test or Fisher exact test where appropriate. In the multivariate analysis, we used Akaike Information Criterion (AIC) to determine the best-fitting genetic model (dominant, co-dominant or recessive).^{23,24} The model with the lowest AIC reflects the best balance of goodness-of-fit and parsimony. We finally coded the genotypes of the –389 G/A promoter polymorphism as a dummy variable according to the hypothesis for a recessive model, that is AA versus AG + GG genotype. The effects of this variable on the disease were estimated by multiple logistic regression models adjusted for age and *APOE* $\epsilon 4$ allele status. Due to its location on chromosome X, *OTC* genetic analyses were done separately in men and women. Extended haplotype frequencies of the different markers were estimated using the Thesias software. The objective of the thesias software is to perform haplotype-based association analysis in unrelated individuals.²⁵ Three independent tests (analyses of two polymorphisms and gender stratification) have been performed and consequently, following Bonferroni corrections, the significant *P*-value threshold has been set at 0.017.

Results

Levels of gene expression were assessed in total RNA from post-mortem brain tissue of 12 controls and 12 AD patients. In the present study, the brain expression profile in each of the AD patients was compared to a pool of the control samples to minimize the influence of individual variability in controls. Among the 106 genes differentially expressed in the initial set of 2741 genes studied (threshold of selection, $P < 10^{-5}$), we selected the *OTC* gene for further analyses because it was the one exhibiting the more pronounced differential expression in the brains of AD cases compared with controls. In fact, no signal at

all for *OTC* was detected in the control pool, whereas specific hybridization was observed in all 12 AD samples. This gene is located on chromosome X where 12 genes were found to be differentially expressed (Table 1).

Using the same brain samples used in the transcriptomic experiments, we first confirmed by RT-

PCR experiments that *OTC* gene was expressed in the frontal cortex of all AD patients but not in 8 out of 9 control brains (Figure 1). Since *OTC* encodes a key enzyme in the urea cycle, which is not functional in 'normal' brain,^{26,27} we searched for expression of other genes in the urea cycle. We were able to detect expression of each of them in all AD brains and

Table 1 Differentially expressed genes in the chromosome X locus

Gene name	Chromosome location	Reference number	Level of variation expression	P
GRPR	p22.2–p22.13	NM_005314	-6.7 ×	4 × 10 ⁻¹³
U2AF1RS2	p22.1	NM_005089	-3.7 ×	4 × 10 ⁻¹¹
MGC4825	p22.11	NM_024122	+2.5 ×	5 × 10 ⁻⁶
OTC	p21.1	NM_000531	+29.1 ×	9 × 10⁻¹¹
NDUFB11	P21.3	NM_019056	-3.2 ×	2 × 10 ⁻¹⁰
PFC	p11.3–p11.23	NM_002621	-2.6 ×	3 × 10 ⁻⁹
PLP2	p11.23	NM_002668	-12.2 ×	2 × 10 ⁻¹⁶
CCDC22	p11.23	NM_014008	-2.9 ×	3 × 10 ⁻⁹
GATA1	p11.23	NM_002049	-2.2 ×	7 × 10 ⁻⁸
SSX1	p11.23–p11.22	NM_005635	-5.6 ×	4 × 10 ⁻¹¹
ABCD1	q28	NM_000033	-3.6 ×	3 × 10 ⁻⁹

Abbreviation: OTC, ornithine transcarbamylase.

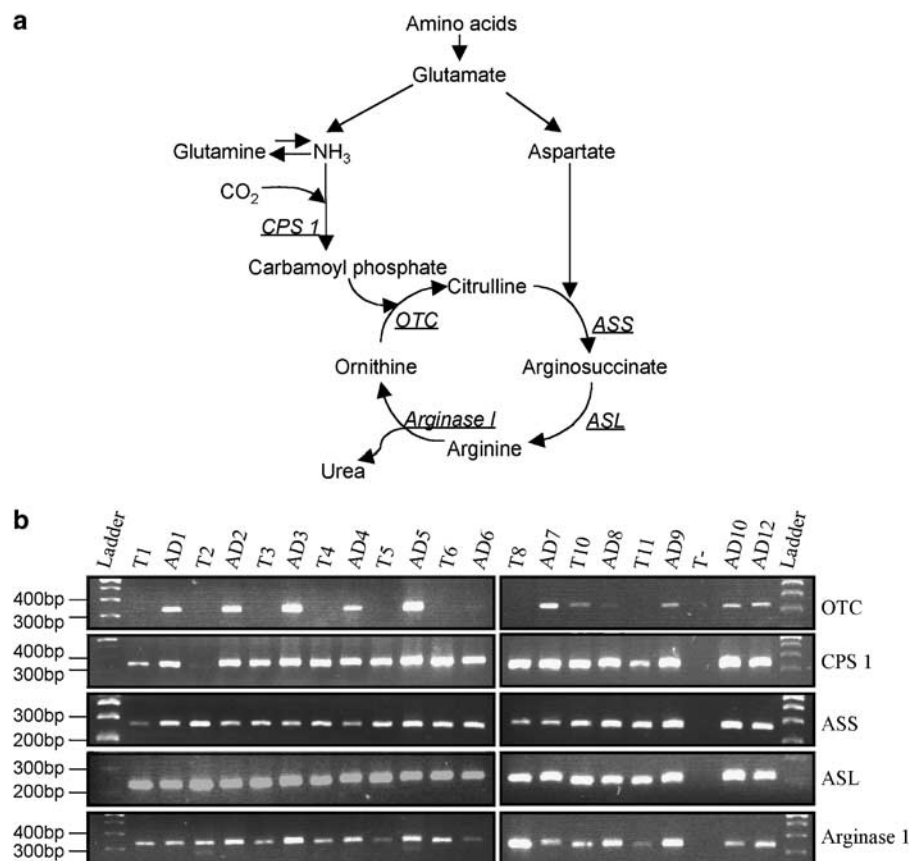


Figure 1 Expression of the enzymes of the urea cycle in the brain. **(a)** Schematic representation of the urea cycle. **(b)** RT-PCR experiments. Total RNA was extracted from the brain of 11 Alzheimer's disease (AD) cases and 9 controls (T) used for the transcriptomic analysis. A control was done by omitting the RNA sample (T-). The primers sets used for the experiments and the size of the expected RT-PCR products are listed in Supplementary Table S2 (Supplementary material).

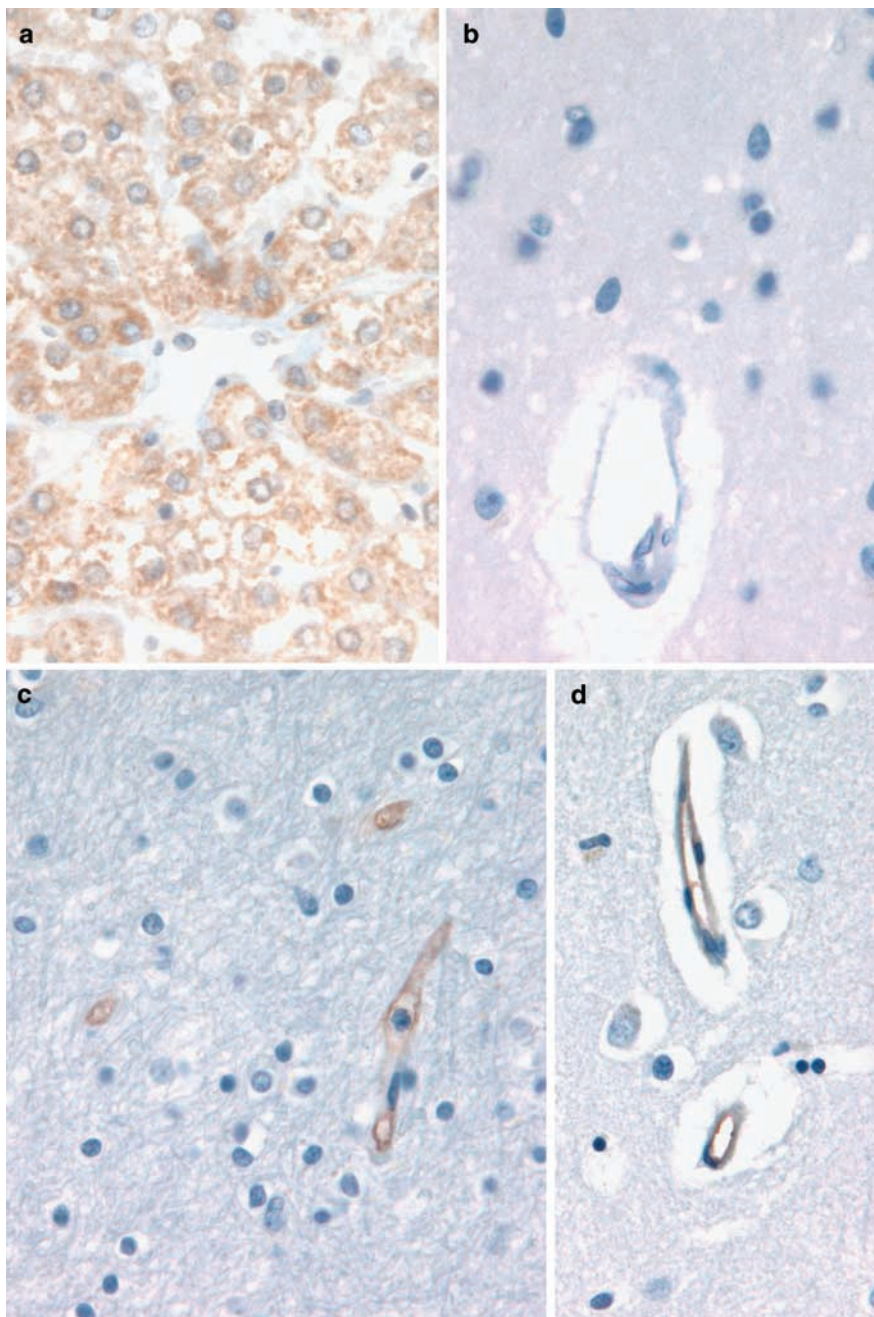


Figure 2 Immunohistochemistry experiments. **(a)** Normal hepatocytes (positive control). The cytoplasm was intensely stained brown by the anti-OTC antiserum but not the vessels and the intrahepatic bile ducts. **(b)** Cortex of control brain; not immunoreactive for OTC. **(c and d)** cortex of AD brain. The endothelium of the brain cortex was labelled by this antibody in 6 out of 12 Alzheimer patients.

controls (Figure 1). As a consequence, in controls, even if the other enzymes of the urea cycle are expressed, the urea cycle cannot be active since OTC, the key enzyme of this cycle, is not expressed. By opposition, this cycle may be active in the brain of AD cases since OTC is present as well as all the other enzymes of the urea cycle.

Using different brain samples from those used in the transcriptomic experiments, we similarly ob-

served that the OTC protein was detected in the brain of AD cases but not in controls. Indeed, the endothelium of capillaries displayed immunoreactivity towards a human polyclonal anti-OTC antibody in the cortex of 6 out of 12 AD cases (Figures 2c and d), whereas no labelling at all could be detected in 4 control brains (Figure 2b). As expected, in control liver sections, a strong signal was observed in hepatocytes but not in the vessels and the intrahepatic

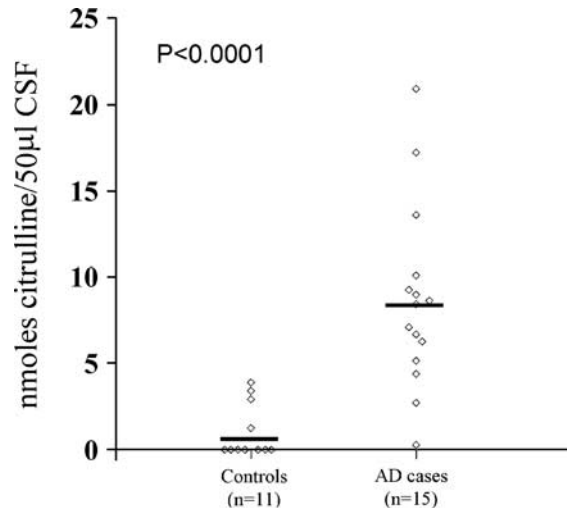


Figure 3 Measures of OTC activity in the cerebrospinal fluid (CSF) of 15 probable Alzheimer's disease (AD) cases and 11 controls without central nervous system diseases. Bold line indicated average. (AD cases: 8.8 ± 5.3 and controls: 1.0 ± 1.6). $P < 0.0001$, Non-parametric Mann-Whitney test.

bile ducts (Figure 2a). No signal was observed when the pre-immune rabbit serum was used instead of the anti-OTC antibody on all the samples tested (data not shown). This observation suggested that the OTC expression may be restricted to vascular endothelial cells in AD. This observation is important and coherent since vascular cerebral endothelial cells are already known to express a complete nitric oxide (NO)-citrulline cycle (including ASS, ASL and arginase I), conversely to others cells of the central nervous systems.^{25,28,29} In AD brains, the OTC expression in the endothelial cells may in consequence lead to a complete and functional urea cycle, as only observed in hepatocytes.

Following these observations, we hypothesize, we may detect an OTC activity in the CSF of AD patients. In order to assess this possibility, we develop an OTC activity assay as previously described. We observed that the OTC activity was 880% increased in the CSF of 15 probable AD cases compared with 11 controls (Figure 3, $P < 0.0001$). OTC activity was detected in the CSF of 14 out of 15 AD (93%) cases whereas no activity at all was detected in the CSF of 7 out of 11 controls (64%). The CSF OTC activity in the 4 others controls was furthermore low. The OTC activity in AD cases and controls was independent on age (data not shown). However, we observed that this OTC activity tended to be higher in women than those in men in both AD cases and controls (respectively, 9.8 ± 5.5 versus 6.3 ± 4.1 , +56% and 1.7 ± 2.0 versus 0.8 ± 1.2 , +112%).

To approach and reinforce a potential implication of OTC in the AD process, we finally assessed whether the *OTC* gene might be a genetic determinant of AD. We searched for polymorphisms within this gene using the NCBI international database ([http://](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snip)

www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snip). Numerous mutations were described, most being responsible for *OTC* deficiency disease. We selected six SNPs within the promoter region, and two other non-synonymous SNPs (Supplementary Figure S1). We were not able to detect the -146 C/T and -69 C/T SNPs in 184 healthy old people. Furthermore, the Glu270Arg SNP exhibited a low frequency (2.3%) and consequently was excluded from further analyses. The remaining five selected SNPs were all in strong LD (Supplementary Figure S1). We finally investigated the -389 G/A and -241 A/G SNPs within the *OTC* promoter. These two SNPs potentially destroyed or created a CpG motif, suggesting a potential modification of the promoter methylation status, such modifications being particularly relevant for the control of gene expression. We evaluated the effect of these two SNPs on the risk of developing AD using a French case-control study including 583 sporadic AD cases and 639 controls. Because the *OTC* gene is located on the X chromosome, Hardy-Weinberg equilibrium could only be tested in women. Departure from Hardy-Weinberg equilibrium was not observed for any SNP. The genotypic distribution of the -389 G/A SNP was significantly different between AD and control samples in women ($P = 0.015$) but not in men (Table 2). Women bearing the -389 AA genotype had an increased risk of developing AD (OR = 2.3, 95% CI (1.3–4.1), $P = 0.005$; this effect was independent of the *APOE* $\epsilon 4$ status). It is important to note that the allelic ORs (-389 A versus G) were similar between men and women (respectively, OR = 1.2, 95% CI (0.8–1.8), NS and OR = 1.2, 95% CI (1.0–1.6), NS) and tended to reach a significant level when both sub-populations were grouped together (OR = 1.2, 95% CI (1.0–1.6), $P = 0.05$). The -241 A/G SNP was not associated with the risk of developing AD whatever the gender (Table 2). Haplotype frequencies were computed from unphased genotypes using the Thesias software in women or directly in men (Table 3). The most common $G_{-389}-A_{-241}$ haplotype was defined as a reference. We observed that the rare $G_{-389}-G_{-241}$ haplotype was associated with decreased risk of developing AD (OR = 0.3, 95% CI (0.1–0.6), $P = 0.0005$). Conversely, the rare $A_{-389}-A_{-241}$ haplotype was associated with increased risk of developing AD (OR = 3.4, 95% CI (1.4–8.8), $P = 0.003$).

In order to evaluate the potential biological relevance of the -389 G/A and -241 A/G SNPs, we investigated whether these SNPs may modify the methylation status of the *OTC* promoter (as suspected by their location in the promoter). The rare A allele of the -389 G/A SNP destroys a CpG motif. The methylation status of the cytosine residue within the CpG and CpA motifs at position -389 was determined by direct sequencing of cloned PCR products amplified from bisulphite-treated genomic DNA. Representative sequencing electrophoregrams are shown in Figure 4. The cytosine in the CpG motif at position -389 was systematically methylated

Table 2 Allele and genotype distributions for *OTC* –389 G/A and –241 A/G SNPs in men and in women

Men	n	Allele distribution ^a (%)				
		G	A			
–389 G/A						
Control	232	172 (0.74)	60 (0.26)			
AD cases	215	153 (0.71)	62 (0.29)			
–241 A/G						
Control	232	167 (0.72)	65 (0.28)			
AD cases	215	158 (0.73)	57 (0.27)			
Women	n	Allele distribution (%)		Genotype distribution (%)	Genotype distribution (%)	Genotype distribution (%)
–389 G/A						
		G	A ^a	GG	AG	AA*
Control	407	615 (0.76)	199 (0.24)	227 (0.56)	161 (0.39)	19 (0.05)
AD cases	368	526 (0.71)	210 (0.29)	195 (0.53)	136 (0.37)	37 (0.10)
–241 A/G						
		A	G ^a	AA	AG	GG ^a
Control	407	595 (0.73)	219 (0.27)	210 (0.52)	175 (0.43)	22 (0.05)
AD cases	368	532 (0.72)	204 (0.28)	194 (0.53)	144 (0.39)	30 (0.08)

Abbreviations: AD, Alzheimer's disease; *OTC*, ornithine transcarbamylase; SNPs, single nucleotide polymorphisms.

^aNS, non-significant. **P* < 0.015.

Table 3 Haplotype distribution from the –389 G/A and –241 A/G SNPs in men, women and in all the population

Haplotype (–389/–241)	Haplotype distribution (%)		
	Controls	AD cases	OR (95% CI)
Men^a			
G-A	167 (0.72)	151 (0.70)	1
A-A	—	7 (0.03)	+ ∞ ^d
A-G	60 (0.26)	56 (0.26)	1.1 (0.7–1.7) ^e
G-G	5 (0.03)	1 (0.01)	0.2 (0.0–1.1) ^f
Women^b			
G-A	587 (0.71)	519 (0.70)	1
A-A	7 (0.01)	14 (0.02)	2.3 (0.9–6.2) ^f
A-G	192 (0.24)	196 (0.27)	1.1 (0.9–1.4) ^e
G-G	28 (0.03)	7 (0.01)	0.3 (0.1–0.7) ^g
All^c			
G-A	754 (0.72)	670 (0.70)	1
A-A	7 (0.01)	21 (0.02)	3.4 (1.4–8.8) ^g
A-G	252 (0.24)	252 (0.27)	1.1 (0.9–1.4) ^e
G-G	33 (0.03)	8 (0.01)	0.3 (0.1–0.6) ^h

Abbreviations: AD, Alzheimer's disease; CI, confidence intervals; OR, odds ratio; SNPs, single nucleotide polymorphisms.

Global test: ^a*P* < 0.03, ^b*P* < 0.001, ^c*P* < 0.0002; haplotype ^d*P* < 0.006; ^eNS, non-significant; ^f*P* = 0.07 (Fischer exact test in men); ^g*P* < 0.003; ^h*P* < 0.0005.

whereas the cytosine in the CpA motif at the same position was not. We similarly assessed whether the –241 A/G SNP may modify the methylation status of the *OTC* promoter as the rare G allele creates a CpG

motif. The cytosine in the CpA motif at position –241 was systematically not methylated whereas the methylation of the cytosine in the CpG motif varied (either methylated or not methylated). All these observations indicated that the methylation status of the *OTC* promoter may be dependent on the –389 G/A and –241 A/G SNPs. Interestingly, the rare G_{–389}–G_{–241} haplotype was associated with a decreased risk of developing AD and may correspond to a high level of methylation of the *OTC* promoter conversely to the rare A_{–389}–A_{–241} haplotype, increasing the risk of developing AD and potentially associated with a lower level of methylation.

Discussion

Combining a genomic and transcriptomic approach, we characterize potential induction of a new metabolic pathway—the urea cycle—in the brain of AD cases. This observation was supported by the identification of the *OTC* protein, a key enzyme of the urea cycle, in endothelial cells of AD brain vessels. Moreover, polymorphisms located within the promoter region of *OTC* may be associated with the risk of developing AD. This last observation nevertheless clearly necessitates further investigations. It will be necessary to validate this present association with AD in other large case-control studies. Finally, *OTC* may not be the only gene that explains the genetic linkage observed on chromosome X in the genome scan studies; other genetic determinants in this chromosome may be implicated.

Once linkage studies have highlighted location of genes potentially associated with a disease, it remains

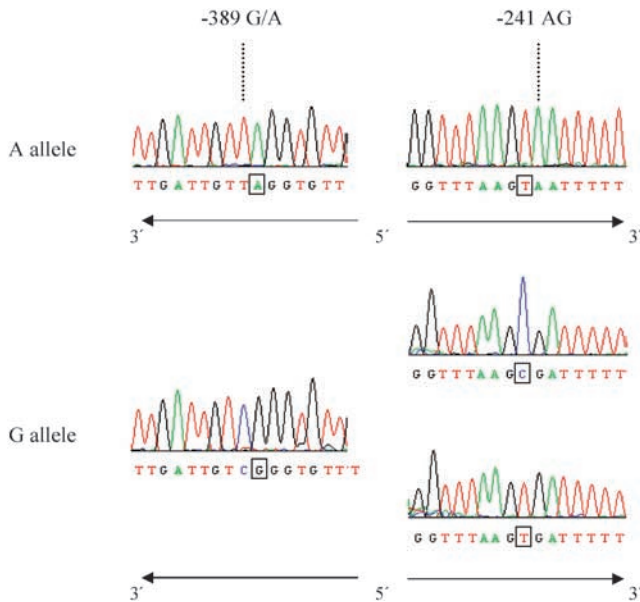


Figure 4 Example of electropherogram of the methylation status within the *OTC* promoter at position -389 and -241 . The position of the -389 and -241 alleles are indicated by the line. The box indicated after bisulphite sequencing either the absence (A) and presence (C) of methylation accordingly to the -389 SNP allele or the absence (T) and presence (C) of methylation accordingly to the -241 SNP allele. The -241 A allele was found to be associated with variable pattern of methylation.

difficult to identify candidates within these broad chromosomal regions. The combination of genome scan studies with more functional approach like differential expression between normal and pathological tissues may help to pick up relevant candidate genes without *a priori* hypotheses about any biological functions or involvement in the AD process. This strategy, successful to identify several genes in rare autosomal recessive disorders,^{30,31} have already been developed to hunt new genetic determinants in multifactorial diseases such as AD. For instance, it has been suggested that genetic variants within the Glutathione-S-transferase omega-1 gene, differentially expressed in the brain of AD cases compared with controls and located on chromosome 10q, may modify age-at-onset of AD.⁷ However, a limitation of these studies was the use of commercial microarrays with genes not necessarily exhaustively covering the linkage peaks identified by genome scanning. To circumvent this limit, we decided to specifically analyse the expression of most of the genes located under known AD linkage peaks using a customized microarray. Supporting our strategy, crosschecking between the genes we selected and affymetrix U133A (one of the most used commercial microarray around the world) indicated that only 48% of the genes were in common.

The differential expression of genes detected by microarray technology between normal and pathological tissues provides a powerful clue for their

potential altered functions. However, several major issues had to be noted: (i) various factors, including for instance mRNA quality or severity of the disease may strongly affect the validity of the results; (ii) the microarray analysis generates a vast amount of data which needs careful interpretation. To take into account these issues, we extracted total RNA from two large banks of brain tissues in order to select the best total RNA samples and we corrected for multiple testing by the selection of genes exhibiting a highly significant differential expression ($P < 10^{-5}$).

Using this strategy, we finally selected a pool of 106 genes differentially expressed in the brain of AD cases compared with controls. At this stage, we have focused on the *OTC* gene, an enzyme of the urea cycle. Indeed, the over-expression of this gene in the brain in AD was intriguing since its expression is usually absent in brain.^{26,27} By RT-PCR, we confirmed the mRNA *OTC* over-expression in AD and noted that all other genes coding for the enzymes of the urea cycle were likewise expressed in our AD brain samples. Consistently with the mRNA expression data, we detected OTC protein in several AD cases but not in controls using immunohistochemistry analyses. Interestingly, these brains were independent of those used in the transcriptomic experiments (see the Materials and methods section). However, at this stage, it is important to note that we were not able to detect a positive reaction with an anti-human OTC antibody in all the tested AD brains. This apparent discrepancy with RT-PCR results may be explained by: (i) a lack of sensibility of the immunohistochemistry analysis; (ii) a low level of expression of the OTC gene in certain AD cases (as observed in the RT-PCR experiments for instance, see AD6 and AD8 samples in Figure 1); (iii) heterogeneity between AD cases. Nevertheless, we systematically observed that the OTC expression was restricted to the vascular endothelial cells. Furthermore, we obtained highly coherent results in GSF of AD patients compared with controls, indicating that OTC expression is strongly increased in the brain of AD cases.

To sustain the potential involvement of the urea cycle in the AD process, we reported that *OTC* may be a minor genetic determinant of AD. Association of the protective $G_{-389}-G_{-241}$ and deleterious $A_{-389}-A_{-241}$ haplotypes were similar either in men or in women, indicating a potential homogeneous impact whatever gender. Interestingly, both SNPs we investigated could be functional. Indeed, the -389 G/A -241 A/G SNPs may modulate the level of methylation of the *OTC* promoter, suggesting a potential modification of *OTC* expression levels. Supporting this hypothesis, the rare $G_{-389}-G_{-241}$ and $A_{-389}-A_{-241}$ haplotypes have opposite effect on the risk of developing AD and, respectively, correspond to high or low levels of methylation of the *OTC* promoter. However, as previously mentioned, nonetheless, and notwithstanding the significant and consistent effects we have observed, additional genetic studies involving

prospective cohorts as well as both family-based and large case-control samples will be required to confirm or invalidate whether the OTC gene is a genetic determinant of AD.

Despite the limited impact of *OTC* as a putative genetic determinant in terms of attributable fraction of risk, our observations reinforce the potential implications of the urea cycle in AD. The reasons for the induction of a complete urea cycle in the vascular endothelial cells of AD brains remains to be elucidated. One of the main functions of the urea cycle is to eliminate ammonia resulting from the degradation of proteins and nucleic acids in order to prevent toxic accumulation.^{26,27,32} As previously described, this urea cycle normally exclusively takes place in liver while in brain, excessive ammonia is usually and rapidly removed by formation of glutamine.²⁷ However, patients with AD have been reported to have significantly lower brain glutamine synthetase (GS) activities than age-matched controls.³³ In parallel, ammonia formation may be increased in the brain in AD following hydrolysis of proteins or deamination of aminopurines and aminopyrimidines.³³ Collectively, present data and that of other studies may implicate an impairment of brain ammonia metabolism in AD. Moreover, in this context it has been reported that ammonia levels are increased in blood and brain of patients with AD.^{34–36} We may postulate that the urea cycle is activated following increased *OTC* gene expression under certain pathological conditions in order to compensate for both decreased GS activity and increased ammonia concentration. Another main function of the urea cycle is to participate in the production of NO via synthesis of arginine, the substrate for nitric oxide synthase.²⁶ There is increasing evidence that NO may have a role in AD pathogenetic mechanisms, either as a neurotoxic or neuroprotective agent.³⁷ The ability of NO to exert cellular damage due to its reactive oxidative properties may be considered as a primary neurotoxic mechanism. On the other hand, NO is likely involved in neuroprotection.³⁸ For instance, as a powerful vasodilator, NO may compensate local hypoperfusion,³⁹ as observed in the brain of AD cases.

Finally, the observation that OTC is expressed in neo-natal brain,²⁶ not in normal adult brain but potentially again in pathological brain as indicated by our results, may reinforce the cell cycle hypothesis in neurodegenerative diseases.^{40,41} The cell cycle hypothesis proposes that either mitogenic signalling, or cell cycle control, or both, are deranged with respect to neurons within the brain of individuals with AD. Loss of the differentiation state and unscheduled re-entry into the cell cycle would be one hallmark of neuronal apoptosis. Such loss of differentiation may induce variations in gene expression. An example is the *BRCA1* gene which have been recently described to be over-expressed in AD: *BRCA1* is expressed in dividing neuronal cells during development but is present in smaller amounts in fully differentiated cells.⁴² Such a loss of differentia-

tion was at this time only described in neurons and induction of the *OTC* gene in endothelial vascular cells may be a first indication of a similar mechanism in another cell type in AD.

In conclusion, we report, for the first time, induction of *OTC* expression in AD process and a potential implication of *OTC* as genetic determinant of AD. This study suggests that disturbance of ammonia metabolism or NO synthesis may be potentially involved in the pathogenesis of AD and this might offer novel therapeutic approaches. Furthermore, our observations indicate that measurement of an OTC activity in CSF may be useful for diagnosis (Figure 3).

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)