

ORIGINAL RESEARCH ARTICLE

# Regulatory region variability in the human presenilin-2 (PSEN2) gene: potential contribution to the gene activity and risk for AD

N Riazanskaia<sup>1\*</sup>, WJ Lukiw<sup>2,3</sup>, A Grigorenko<sup>1,4</sup>, G Korovaitseva<sup>1</sup>, G Dvoryanchikov<sup>1</sup>, Y Moliaka<sup>1</sup>, M Nicolaou<sup>5</sup>, L Farrer<sup>5,6</sup>, NG Bazan<sup>2,3</sup> and EI Rogae<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Brain Genetics, Research Center of Mental Health, Russian Academy of Medical Sciences of Russia, Moscow 113152, Russia; <sup>2</sup>Neuroscience Center and <sup>3</sup>Department of Ophthalmology, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112–2272, USA; <sup>4</sup>Institute of Molecular Genetics, Russian Academy of Sciences, Moscow; <sup>5</sup>Department of Medicine (Genetics Program) and Dept of Neurology, Boston University School of Medicine, Boston, MA, USA; <sup>6</sup>Department of Epidemiology and Biostatistics, Boston University School of Public Health, Boston, MA, USA

We have analyzed the 5'-upstream promoter region of the presenilin 2 gene (PSEN2) for regulatory elements and examined Alzheimer disease (AD) patients and non-demented individuals for polymorphisms in the 5' upstream promoter region of the PSEN2 gene. Direct sequencing analysis detected a common single adenine (A) nucleotide deletion polymorphism in the upstream promoter region of the PSEN2 gene. Examination of cohorts of AD patients and age-matched control individuals revealed no statistically significant differences in the frequency of this polymorphism when compared with the total sample of AD patients and control individuals. However, subgroup and regression analysis suggested that the relatively rare –A/–A genotype increases risk of AD among subjects lacking apolipoprotein E (APOE)  $\epsilon$ 4 and among persons ages 65 years and younger. DNA sequence and DNA-protein binding analysis demonstrated that this mutation negates binding with putative repressor transcription factor (TF), interferon regulatory factor 2 (IRF2), in nuclear extracts prepared from the aged human brain neocortex. However this mutation creates a potential regulatory element, C/EBPbeta, that is responsive to pro-inflammatory (PI) induction. The expression activity assay with luciferase reporter gene into normal human neural progenitor cells in primary culture shows that the mutant PSEN2 regulatory region exhibits a 1.8-fold higher level of basal expression and is sensitive to IL-1 $\beta$  and A $\beta$ 42, but that it is synergistically induced 3.2-fold over the wild-type PSEN2 by [IL-1 $\beta$ +A $\beta$ 42]. These results suggest that under PI and oxygen stress conditions relatively minor variations in PSEN2 promoter DNA sequence structure can enhance PSEN2 gene expression and that consequently these may play a role in the induction and/or proliferation of a PI response in AD brain.

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## Introduction

Presenilin-1 (PS1 or PSEN1; AD3, chr14q24.3) and presenilin-2 (PS2 or PSEN2; AD4; chr1q42.1) were initially found via the strategy of positional cloning to be a novel family of genes bearing mutations in familial AD (AD) and encoding polytopic integral transmembrane proteins.<sup>1,2</sup> Missense-mutations in the PSEN1 are a relatively frequent cause of early-onset familial AD.

The frequency of mutations in the PSEN1 gene in AD or remains to be determined but may account for 18–50% of familial early-onset AD or even up to 70–90% of AD with proven history of autosomal dominant inheritance in large pedigrees.<sup>1–7</sup> A recent analysis of mutations in the PSEN1 gene in a non-referral series of AD patients and asymptomatic persons with family history of AD revealed that about 11% of AD in such a series may be explained by mutations in the PSEN1 gene. Thus, the clinical screening for mutations in encoding regions of the PSEN1 gene, in particular in individuals with a family history of AD before 60 years may be successful for diagnostic testing.<sup>6</sup> Missense-mutations in homologous PSEN2 gene are rare. Two mutations were documented in early- and late-onset AD cases in two large pedigrees of Italian and Volga-

Correspondence: E Rogae, BNRI, University of Massachusetts Medical School, 303 Belmont Street, Worcester, MA 01604, USA. E-mail: Evgeny.Rogaev@umassmed.edu or Research Center of Mental Health, Moscow. E-mail: Rogae@dol.ru  
\*N Riazanskaia and W Lukiw contributed equally to this work.  
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German origin. More recently, four other mutations were reported in single cases with AD.<sup>1–3,8</sup> Whether non-coding polymorphisms in the PSEN1 gene also contribute to risk for sporadic and/or late-onset AD has not yet been clearly determined. As in many association studies the initial reports of intronic polymorphism (intron 8 numbering<sup>7</sup> or intron 9 numbering<sup>9</sup>) linked to AD have been replicated in some but not confirmed in many other studies.

It was demonstrated that PSEN1 and PSEN2 are key regulators of endoproteolysis or unusual proteases themselves with intra-membranous enzyme activity. PSEN1 and PSEN2 participate in cleavage of the mammalian Notch 1 cell surface receptor in *Drosophila* and *Caenorhabditis elegans*,<sup>10,11</sup> which eventually leads to transcriptional modulation of genes involved in cell differentiation control. In addition to the PSEN1 and PSEN2 functions, known to be involved in cell fate decision during neural development, these proteins are implicated in the novel nicastrin-mediated intra-membranous processing of beta-amyloid precursor protein ( $\beta$ APP) into amyloidogenic  $\beta$  peptides.<sup>12,13</sup> AD-associated missense mutations facilitate APP cleavage into a  $\gamma$ -secretase site, which may contribute to the accumulation of the most amyloidogenic 42-aminoacid amyloid derivative.<sup>10–12,14</sup> It is conceivable that elevated expression of presenilins, as well as missense mutations in these genes, may increase  $\gamma$ -secretase cleavage and the amount of 42 amyloid.

PSEN1 gene expression may be induced in animal models of glaucoma, by  $\beta$  or IL-1 $\beta$  or by retinoic acid in cultured brain cells,<sup>15–17</sup> but the constitutive levels of expression of the PSEN1 gene are relatively equal in most human tissues and brain regions. The presenilin 2 gene, however, demonstrates a remarkably different expression in human tissues<sup>2</sup> and may be much more effectively regulated by different inducible factors than the PSEN1 gene. For example, we have shown that there are multiple hypoxia inducible elements in the PSEN2 5'-upstream promoter region and a sustained increase in PSEN2 gene expression in rat pup retina after induction by hypoxia.<sup>18,19</sup> The fact that PSEN2 RNA message was found in the human brain neocortex to have a relatively long half-life (>12 h) suggested that genetic factors involved in PSEN2 RNA message generation may be functionally important.

It would be of interest to identify polymorphisms in the 5'-regulatory regions of PSEN1 and PSEN2 and to elucidate whether such polymorphisms could contribute to more common forms of AD. Recently, polymorphisms in the 5'-PSEN1 promoter regions were identified, including -48C  $\rightarrow$  T, which demonstrated marginally significant association with early-onset AD in a case-control study in the Dutch population.<sup>20,21</sup> The increased genetic risk for AD and correlation with  $\beta$  load in brain in patients with -48C/C homozygous genotype were reported in the British population.<sup>22</sup> In another independent study, we identified the same polymorphism in the 5'-PSEN1 region, but found no association with AD in the Russian population (in preparation). To date no data have been reported for

analyzing polymorphisms in the promoter region of PSEN2 gene in AD.

In this study we examined the possibility that mutations in the human PSEN2 promoter may be responsible for elevated PSEN2 expression and the risk for dementia in AD patients vs age-matched controls. Using DNA sequencing and genetic association analysis, we examined the PSEN2 promoter sequence in the AD patients and corresponding numbers of age-matched control individuals for mutations in the 5' upstream region of the PSEN2 gene. Our screening shows a common nucleotide polymorphism ('A' deletion) in the upstream promoter regulatory region of the PSEN2 gene. This mutation may be sensitive to PI mediators prevalent in AD brain and may be associated with an increased risk for AD.

## Methods

### Screening for polymorphisms in the PSEN2 gene promoter

The standard procedures of PCR, Single Strand Conformation Polymorphism (SSCP) and direct sequencing analysis were used for screening polymorphisms in the upstream promoter regions of presenilin 2 gene. To screen GC-enriched regions, specific PCR and sequencing modifications were applied.<sup>23,24</sup> The initial analysis of the 5'-upstream promoter genomic region of the PSEN2 gene (accession number U50871)<sup>25</sup> in 10 AD and 10 non-demented individuals revealed a polymorphism caused by single nucleotide deletion at the 24914 site. To evaluate this polymorphism further the primers *ps2-e1*: 5'-**taaactgtggcatacatga** and *ps2-e2*: 5'-**ccatacccattgagaagt** were designed for a 278-bp PCR fragment (24781–25058). PCR amplification was carried out in a total volume of 20- $\mu$ l containing 2.5 mM MgCl<sub>2</sub>, 67 mM Tris-HCl pH 8.4, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 200  $\mu$ g ml<sup>-1</sup> BSA, 200  $\mu$ M dNTPs, 0.5 units of Taq-polymerase. Amplification parameters were 30–32 cycles with denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s. The polymorphism was detected by two methods: *Ddel*-digestion of the PCR fragment or direct oligonucleotide hybridization. Digestion of 10  $\mu$ l of PCR product was performed in total volume of 20  $\mu$ l with 2 units of *Ddel* enzyme for 3 h at 37°C. The PCR fragment contains one non-polymorphic and one polymorphic *Ddel* sites (CTNAG). The allele (+A) is digested and allele (-A) is not digested by *Ddel* enzyme at the 24914 site. To confirm that heterozygous genotypes may not be caused by partial indigestion of the PCR products, some genotypes were also re-tested by differential hybridization with oligonucleotides. The PCR products were transferred to duplicate nylon membranes and hybridized with gamma-<sup>32</sup>P labelled '+A' oligonucleotide: 5'-aacatgctaagtgaagac and '-A' oligonucleotide: 5'-aacatgctagtgaagac. Hybridization conditions: 5  $\times$  SSC, 5  $\times$  Denhardt's, 0.5% SDS for 6 h at 47°C; washing: 2  $\times$  SSC, 0.2% SDS at 55°C for '+A' and 50°C for '-A'.

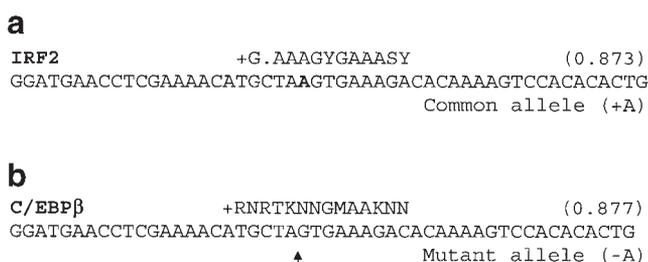
### Subjects and genetic association study

More than 250 patients with dementia were ascertained through the Alzheimer Disease and Related Disorders Center and other clinical departments at the Mental Health Research Center in Moscow. The diagnosis of AD was established according to ICD-10 and NINCDS-ADRDA criteria<sup>26,27</sup> and as we have described.<sup>28,29</sup> The patients underwent a standard neurological examination, a personal interview, psychometric testing and/or brain imaging (CT or MRI). A total of 178 patients met criteria for AD. Patients with vascular dementia or mixed (vascular plus AD) dementia were excluded from the analysis. An ethnically- and age-matched control group of 234 individuals of Russian origin was selected as previously described.<sup>28,29</sup> The study was carried out with the informed consent of the participants as described.<sup>28,29</sup> The allele and genotype frequencies between AD cases and controls were compared by  $\chi^2$  and Fisher exact test in total samples and groups stratified by age (using 65 years as the cutoff) and APOE  $\epsilon 4$  carrier status. Logistic regression procedures were used to evaluate the influence of PSEN2 genotype on the risk of developing AD, adjusted for APOE genotype, age and sex. All statistical procedures were carried out using SAS (Statistical Analysis System) and as described.<sup>28,29</sup>

### Electrophoretic mobility shift assay (EMSA)

The 27 oligonucleotides: wild type 5'-AAA-CATGCTAAGTGAAAGACACAAAAG-3' and mutant type 5'-AAAACATGCTA-GTGAAAGACACAAAAG-3' were synthesized for the gel shift analysis. To preserve % A+T content in each DNA consensus sequence, an extra 'A' residue was added to the 5' end of the mutant consensus.

Five micrograms of nuclear protein extracts (NPXTs) derived from HeLa or NHNP cells as described<sup>30</sup> were incubated with (<sup>32</sup>P-ATP (~3000 Ci mmol<sup>-1</sup>) end-labeled IRF2 consensus and mutant oligonucleotides (Figure 1) in 5  $\mu$ l volumes, and were analyzed on 5% acrylamide/90 mM Tris-borate pH 8.4, 1 mM EDTA (TBE) gels, dried onto 2 mm Whatman filter paper at 80°C for 2 h and phosphorimaged. Gel supershift assays employed the rabbit polyclonal IgG specific for the human IRF-2 (C-19) epitope (Santa Cruz SC-498X).



**Figure 1** Similarity of common PSEN2 allele (wild-type) to IRF2 regulatory factor and mutant allele to C/EBP regulatory factor. Y- C or T; S- C or G; R- A or G; N- A or C or G or T; M- A or C; K- G or T. Arrow shows the deletion of A nucleotide occurring in common human population with frequency 0.2.

### Transient transfection of normal human neural progenitor cells in primary culture

The PCR product fragments spanning the 24781–25058 region from the individuals with +A/+A genotype (wild-type) and -A/-A genotype (mutant) were cloned into the pGL2-Promoter vector (Promega, Madison, USA) with luciferase gene. Clonetics' NHNP (normal human neural progenitor) cell lines were obtained from the LSUHSC Tissue Culture Cell Facility (Joelle Finley and Josephine Rouselle). NHNP cells derived from explanted human fetal tissue (Clonetics) were performance tested and tested negative for HIV-1, hepatitis B and C, mycoplasma, bacteria yeast and fungi. DNA fragments containing human-specific control and PSEN2 regulatory regions cloned into PGL2 luciferase reporter vector (Promega) were transiently transfected into NHNP cells using Lipofectamine-2000 transfection according to the manufacturer's instructions (BRL/Life Technologies). In our hands NHNP cells incorporate pGL2/pGL3 constructs with much higher efficiency using the Lipofectamine-2000 protocol (BRL-Life Technologies) when compared to Lipofectamine-Plus or DOTAP (Boehringer Mannheim) transfection reagent systems.

### Inducers and inhibitors

A $\beta$ 42 and AB40S, purchased from AnaSpec (San Jose, CA, USA), were dissolved in a minimal amount of DMSO (Sigma D-8779) and diluted with water to the desired concentration. IL-1 $\beta$  (human, recombinant; Sigma I-4019) was made up as a  $\mu$ g ml<sup>-1</sup> solution in PBS/0.1% HSA (human serum albumen; Sigma 2-2257). Transfected cells carrying pGL2/pGL3 control plasmids or human PSEN2 wild or deletion mutant-luciferase reporter constructs were serum deprived and treated with ligand/inducers A $\beta$ 42, IL-1 $\beta$  and (A $\beta$ 42+IL-1 $\beta$ ) for 12–24 h before assaying for luciferase activity in the cell lysates. Luciferase assays were performed in replicative experiments ( $n = 9$ ) using 96 well plates, and signals were quantitated using a Lab Systems Fluoroskan FL Fluorescence/Luminescence microplate reader.

### DNA sequence analysis and quantitation

DNA sequence analysis identifying putative *cis*-acting DNA regulatory elements lying between -1800 bp and +100 bp of the human PSEN2 gene promoter (-1800 bp upstream from the transcription start site at +1; corresponding to GenBank U50871 nucleotide 26409 or GenBank NM012486 nucleotide 19)<sup>23</sup> was performed using Hitachi DNASIS software (Version 2.6). Luciferase data were quantitated and figures were generated using Designer Version 6.0 and Excel Version 5.0 (Microsoft). The statistical significance of the luciferase data was analyzed in a two-way factorial analysis of variance ( $P$ , ANOVA) using SAS.

## Results

### The 5'-region of PSEN2

We analyzed the 5'-sequences of cDNAs or ESTs of PSEN2 gene from commercial and publicly available

cDNA libraries and databases and found that, in addition to the 5'-regions reported previously,<sup>2,3</sup> the most 5'-extended transcripts contain about 34 bp (25763–25796 bp in genomic sequence, accession U50871) located at a distance of more than 600 bp from the initially reported start of transcription (26409 bp). Apparently, this fragment represents a novel 5'-untranslated exon (exon 1) (Figure 2). Thus, the 5'-heterogeneity of cDNA clones may indicate two sites of initiation of PSEN2 transcription located upstream and downstream of this exon. Alternatively, it is conceivable that there is a single site of initiation of transcription located upstream of exon 1, which is not identified in most cDNA sequences because of the very short sequence of this exon in the 5'-transcript end. We screened for polymorphism in the 5'-upstream exon1 region of PSEN2 in 10 AD and 10 control subjects by the Single Strand Confirmation Polymorphism method and direct sequencing of PCR fragments using primer oligonucleotides as described in Materials and Methods. One of the sites (position 24914), located near imperfect microsatellite (TG)<sub>n</sub> tract (24800–24822), was polymorphic and represented deletion of –A which may be detected by *Ddel*-digestion. The (TG)<sub>n</sub> tract was non-polymorphic in these individuals.

#### Genetic analysis

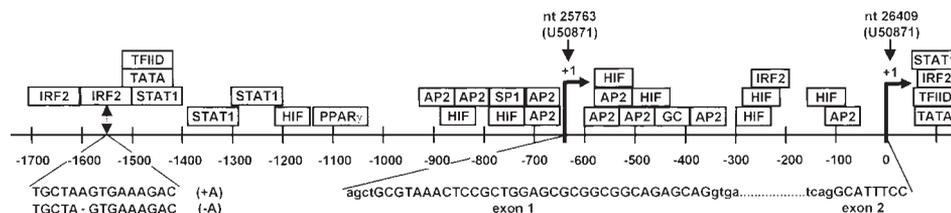
Analysis of the 24914 polymorphism showed that among controls the frequency of the A deletion is 0.2 and the genotype distribution is in Hardy–Weinberg equilibrium. In the total sample, AD was not associated with any PSEN2 genotype or allele (Table 1). However, among subjects lacking the APOE 4 allele, there was an excess of the rare A/A genotype in patients compared with controls ( $P = 0.001$ ). A similar pattern was found in subjects ages 65 years and younger, but this result was only borderline significant ( $P = 0.075$ ). Multivariate analysis (Table 2) showed that the homozygous A deletion genotype increases the odds of AD in persons lacking the  $\epsilon 4$  allele (OR = 10.6, 95% CI = 2.6–43.5).

#### Regulatory elements

To elucidate further whether the identified polymorphism may have biological relevance we initially screened for the potential regulatory sites in 5'-region of the PSEN2. The immediate 5'-upstream region of the exon 1 is GC-enriched suggesting that this region may represent a promoter region of the PSEN2. We indicated multiple common transcription factors AP2 and the signal transduction and activator of transcription factors (STAT1) around each of the potential sites of initiation of transcription. The relatively rare HIF-1-alpha (hypoxia inducible factor 1) sites were numerous in the PSEN2 region, occupying about 1 kb upstream and downstream of exon1. Importantly, the wild type of the polymorphic sequence (+A) is similar to interferon regulatory factor (IRF-2) acting as repressor of transcription.<sup>31</sup> However, the deletion of A nucleotide (–A) diminished the homology to IRF2, but, interestingly, created a new potential regulatory site for transcription factor C/EBP which can be modulated by pro-inflammatory factors (Figure 1). C/EBP (CCAAT/enhancer binding protein) is a heat stable DNA-binding protein that appears to function exclusively in terminally differentiated cells and is known to be induced by proinflammatory signaling factors such as PAF, pro-inflammatory cytokines and lipopolysaccharide.<sup>19</sup>

#### Electrophoretic mobility shift assay (EMSA)

To test the DNA-protein binding activity of the polymorphic region, the oligonucleotides with A deletion (mutant) and without deletion (wild) (Material and Methods) were designed (Figure 1). Gel shift analysis with nuclear protein extracts (NPXT) from HELA cells or human brain neocortex (NCTX) showed protein binding to the wild type of allele with IRF2 recognition site 5'-TGCTAAGTG-3' but strongly reduced or non-existent binding to the mutant IRF2 binding site 5'-TGCTAGTG-3' (Figure 3 a,b). To prove further that this mobility shift is caused by specific binding of this region to IRF2, we performed pre-incubation of the NPXT from adult brain neocortex with specific anti-



**Figure 2** Schematic structure of the human PSEN2 promoter. Schematic layout of the human PSEN2 promoter showing alternate transcription start sites at nt 25763 and nt 26409 (heavy bent arrows) from GenBank accession U50871 and putative transcription factor regulatory sites. The PSEN2 promoter (–A) deletion mutation is shown at the IRF2 site at approximately nt –1560. IRF2 = interferon regulatory factor 2; TFIID = transcription factor II D (which recognizes the TATA box and promotes RNA polymerase II positioning and binding); STAT1 = signal transducer and activator of transcription type 1; HIF = hypoxia inducible factor 1 alpha; PPAR $\gamma$  = peroxisome proliferator-activated receptor type gamma; AP2 = activator protein 2; GC = glucocorticoid responsive element. Note abundance of potential HIF regulatory sites which convey hypoxia sensitivity to gene promoters. Additional transcription factor regulatory sites have been omitted due to space constraints.

**Table 1** PS2 genotype and allele distributions among AD cases and controls stratified by APOE  $\epsilon 4$  status and age

Group	PSEN2 Genotypes			PSEN2 Alleles	
	+A/+A	+A/-A	-A/-A	+A	-A
<b>Total</b>					
Cases	111 (62.4%)	57 (32.0%)	10 (5.6%)	279 (78.4%)	77 (21.6%)
Controls	143 (61.1%)	85 (36.3%)	6 (2.6%)	371 (79.3%)	97 (20.7%)
<b>APOE <math>\epsilon 4</math> (+)</b>					
Cases	78 (66.7%)	37 (31.6%)	2 (1.7%)	193 (82.5%)	41 (17.5%)
Controls	38 (62.3%)	20 (32.8%)	3 (4.9%)	96 (78.7%)	26 (21.3%)
<b>APOE <math>\epsilon 4</math> (-)</b>					
Cases	33 (54.1%)	20 (32.8%)	8 (13.1%)	86 (70.5%)	36 (29.5%)
Controls	105 (60.7%)	65 (37.6%)	3 (1.7%)	275 (79.5%)	71 (20.5%)
<b>Age <math>\leq 65</math> years</b>					
Cases	57 (60.6%)	28 (29.8%)	9 (9.6%)	142 (75.5%)	46 (24.5%)
Controls	75 (62.5%)	42 (35.0%)	3 (2.5%)	192 (80.0%)	48 (20.0%)
<b>Age <math>&gt; 65</math> years</b>					
Cases	54 (64.3%)	29 (34.5%)	1 (1.2%)	137 (81.5%)	31 (18.5%)
Controls	68 (59.7%)	43 (37.7%)	3 (2.6%)	179 (78.5%)	49 (21.5%)

**Table 2** Odds of AD according to PSEN2 genotype adjusted for age and sex

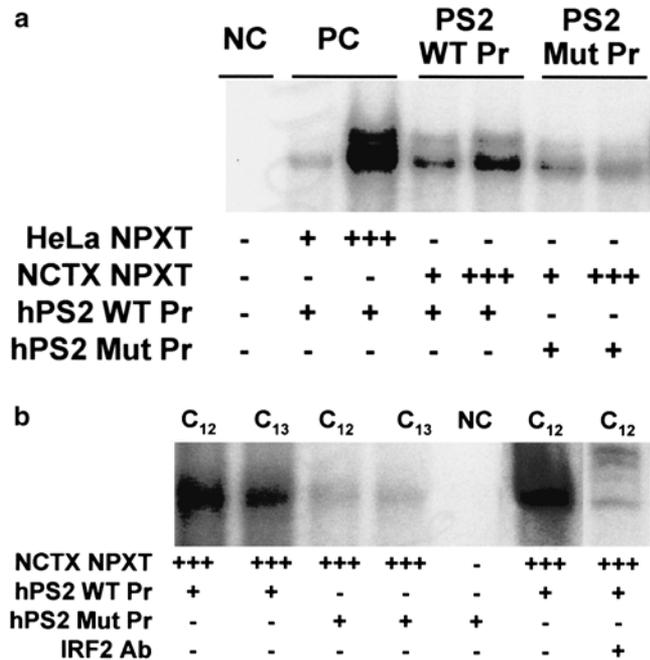
PSEN2 genotype	Number of AD patients	Number of controls	Odds ratio (95% confidence interval)
<b>Total</b>			
-A/-A	10	6	3.4 (1.1–10.5) <sup>a</sup>
+A/+A; +A/-A	168	228	1 (Reference)
<b>APOE <math>\epsilon 4</math> (+)</b>			
-A/-A	2	3	0.31 (0.05–1.9)
+A/+A; -A/+A	115	58	1 (Reference)
<b>APOE <math>\epsilon 4</math> (-)</b>			
-A/-A	8	3	10.6 (2.6–43.5)
+A/+A; -A/+A	53	170	1 (Reference)

<sup>a</sup>Adjusted for APOE  $\epsilon 4$  status.

bodies against IRF2 protein. We found that the binding activity of the NPXT with the wild allele was almost completely abolished (Figure 3b). Thus, the single nucleotide deletion, which mutates from the wild type of an existing IRF2-repressor DNA binding site, leads to impairment of DNA/protein binding to IRF2 repressor of transcription. It may derepress PS2 gene expression in some environments, eg proinflammatory, against a background of brain aging. To examine this possibility we analyzed the transcription activity of wild and mutant alleles in neurones.

#### Luciferase reporter gene assay

We cloned the 5' PSEN2 polymorphic region from both alleles into pGL2 promoter luciferase reporter vector.



**Figure 3** Gel shift assay. (a) Electrophoretic mobility shift assay (EMSA) using HeLa and human neocortex (NCTX) nuclear protein extracts and the human PS2 (hPS2) wild-type (WT) and mutant (Mut) promoter sequences. NC = negative control; PC = positive control; note preferential binding to the human PS2 WT promoter (PS2 WT Pr). (b) EMSA using NCTX nuclear protein extracts derived from two control temporal lobe neocortices (C12 and C13) and the hPS2 WT and Mut promoter sequences. NC = negative control. Rightmost lane shows get supershift using NCTX pre-treated with the IRF2 antibody (Ab).

The constructs with PSEN2 wild-type and mutant promoter regulatory region were transiently transfected. We quantitated basal control and mutant PSEN2 regulatory region luciferase expression levels as well as the relative signal intensities of the luciferase reporter after treatment of NHNP cells with proinflammatory (PI) peptide and cytokine inducers A $\beta$ 42 and IL-1 $\beta$ .

Gene induction from the mutant (-A) PSEN2 promoter-luciferase promoter construct was found to exhibit a 1.8-fold higher level over basal expression of PSEN2 wild-type promoter. This mutant promoter is particularly sensitive to IL-1 $\beta$ , less so to A $\beta$ 42. However, it is particularly induced, 3.2-fold over the control hPS2 promoter construct, by the synergistic combination of (IL-1 $\beta$ +A $\beta$ 42) (Figure 4).

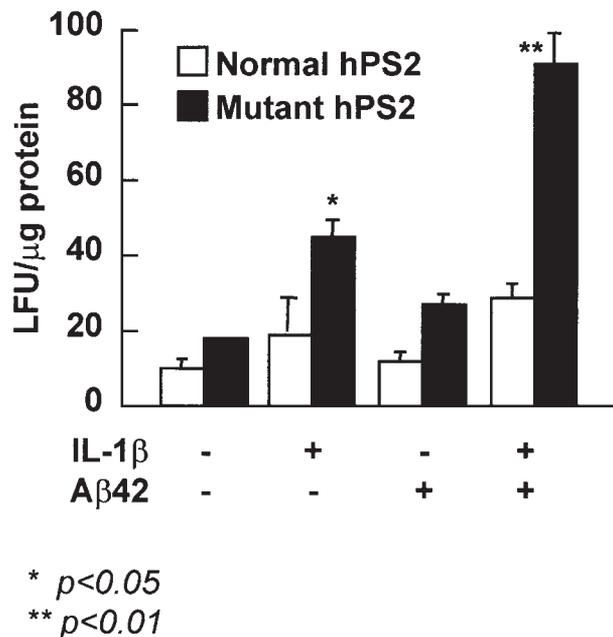
## Discussion

In this study we describe a polymorphism located in the 5'-upstream promoter region of the PSEN2 gene which is modestly associated with AD. The hypothesis is that, in addition to rare missense mutations in familial forms of AD, which may be transmitted as an autosomal dominant trait,<sup>1-3,32</sup> more common polymorphisms may contribute to increased risk for AD. Polymorphisms in the promoter and intronic regions of PSEN1 are reported to be associated with AD.<sup>33</sup> These data, however, were not confirmed in other studies.<sup>34</sup> In our study, for example, we found no associ-

ation of PSEN1 intron 8 polymorphism with early- or late forms of AD in the Russian population.<sup>29</sup> Independently, we identified and analyzed polymorphisms in the PSEN1 promoter which were identical to -48C→T in the 5'PSEN1 region reported by Theuns *et al.*<sup>21</sup> This polymorphism also showed no association with AD in the Russian population group (in preparation).

We previously reported an association of a polymorphism leading to synonymous nucleotide substitution in the PSEN2 gene.<sup>29</sup> This polymorphism might be in linkage disequilibrium with other biologically significant polymorphisms in PSEN2. Because the direct screening of the coding region detected no common polymorphisms causing amino acid substitutions (unpublished data), we searched for variations in the 5'-promoter and upstream promoter region of PSEN2. Screening for polymorphisms in the promoter revealed the single nucleotide deletion in a putative regulatory element of PSEN2. There was no evidence for association between this polymorphism and AD in the total sample. Because the APOE  $\epsilon$ 4 allele, and the  $\epsilon$ 4/ $\epsilon$ 4 genotype in particular, are the most common risk factors for AD in many different Caucasian populations, including the one used in this analysis,<sup>28,29</sup> it may obscure other more minor genetic risk factors. Stratification of subjects by 4 status revealed that the -A/-A genotype may contribute to risk for AD in persons lacking 4. An association was also observed between this genotype and AD occurring before age 65 years, but this result was not significant perhaps due to sample size. Because the -A/-A genotype is relatively rare, these findings should be replicated in other populations comprised of large AD and control samples.

Nevertheless, the genetic data indicating a role of this polymorphism in AD pathogenesis are supported by functional analysis in human neural cells, which demonstrate that the single nucleotide deletion (-A) alters the regulatory elements leading to the abolishment of the DNA protein binding to the transcription repressor of transcription (IRF2). These data suggest that the mutant allele may enhance the transcriptional activity of the PSEN2 gene *in vivo*. The activity of PSEN2 and PSEN1 is linked to endoproteolytic cleavage of the Notch receptor and APP protein within their transmembrane domains and, as a consequence, release of intracellular domains of APP and Notch proteins which potentially function as transcriptional activators.<sup>35,36</sup> Importantly, other products of such cleavage (such as  $\gamma$  secretase proteolysis) of APP result in secreted amyloidogenic A $\beta$ 40 or 42 peptides that accumulate as plaques in AD brains. Although, there was a report of increased A $\beta$ 42 by partial inhibition of PSEN1 by antisense RNA,<sup>37</sup> the inhibition of APP processing and A $\beta$ 40 and A $\beta$ 42 production in PSEN1 or double PSEN1 and PSEN2 deficient mice was demonstrated in many studies.<sup>10-12</sup> It is reasonable to suggest that over-expression of PSEN2 as missense mutations in PSEN2 may facilitate the  $\gamma$ -secretase activity of PSEN2. Interferon (IFN) regulatory factors 1 and 2 (IRF-1 and IRF-2) were initially described as transcriptional regulators of IFN and antiviral activity,



**Figure 4** Transfection into normal human neural progenitor (NHNP) cells using normal and mutant hPS2 constructs with luciferase reporter. Bar graph showing effects of normal and mutant alleles or 5'-PSEN2 regions (hPS2) on reporter gene activation in the presence of IL-1 $\beta$ , A $\beta$ 42 and (IL-1 $\beta$ , A $\beta$ 42). Compared to control, note synergistic induction effect on the mutant hPS2 promoter in the presence of inducers (IL-1 $\beta$ +A $\beta$ 42). LFU = luciferase units. \* $P < 0.05$ ; \*\* $P < 0.01$  (ANOVA).

cell growth and differentiation. The transcriptional activator IRF-1 and transcriptional repressor IRF-2 function as competitors for virtually identical DNA binding sites.<sup>31</sup> However, it is possible that, in the absence of stimulation, the IRF2 protein, which is more abundant in neural cells, predominantly occupies DNA/protein sites inhibiting transcription. The mutation in this regulatory region may contribute to a slight up-regulation of the transcription. Importantly, using wild and mutant alleles of 5'-upstream of PSEN2 promoter (+A or -A) cloned into pGL2 luciferase-reporter vectors and expressed into neuronal NHNP cells in culture, we found that inflammatory factors increase the activity of the luciferase reporter gene via the mutant (-A) allelic sequence.

In summary we speculate that the identified variation in the PSEN2 promoter may affect PSEN regulation because: (a) 'A' (adenine) nucleotide deletion has the relatively strong influence on DNA bending, and binding of the 5'-PSEN region to nuclear protein, possibly that for the repressor IRF2; and (b) this mutation may increase sensitivity of this regulatory region to pro-inflammatory factors. While this promoter variation in its homozygous status is relatively rare, it shows for the first time that relatively slight changes in PSEN2 promoter DNA sequence can potentially accelerate PSEN2 gene expression in the presence of pro-inflammatory inducers such as IL-1 and A peptide. Promoter DNA mutations/polymorphisms may add or delete a critical transcriptional factor-DNA binding site, which becomes activated only during brain aging or in a pro-inflammatory environment. PSEN2 promoter mutation gain-of-function for PSEN2 gene expression may therefore be important in driving aberrant processing of  $\beta$ APP into A $\beta$  peptides (Figure 1) and thereby further fuel pro-inflammatory signaling in AD brain.

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