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# Genetic and biochemical studies of SNPs of the mitochondrial Aβ-degrading protease, hPreP

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ABSTRACT

Several studies suggest mitochondrial dysfunction as a possible mechanism underlying the development of Alzheimer disease (AD). There is data showing that amyloid-β (Aβ) peptide is present in AD brain mitochondria. The human presequence protease (hPreP) was recently shown to be the major mitochondrial Aβ-degrading enzyme. We investigated if there is an increased susceptibility to AD, which can be attributed to genetic variation in the hPreP gene *PITRM1* and if the proteolytic efficiency of recombinant hPreP variants is affected. When a total of 673 AD cases and 649 controls were genotyped for 18 single nucleotide polymorphisms (SNPs), no genetic association between any of the SNPs and the risk for AD was found. In contrast, functional analysis of four non-synonymous SNPs in hPreP revealed a decreased activity compared to wild type hPreP. Using Aβ, the presequence of ATP synthase $F_1\beta$ subunit and a fluorescent peptide as substrates, the lowest activity was observed for the hPreP(A525D) variant, corresponding to rs1224893, which displayed only 20–30% of wild type activity. Furthermore, the activity of all variants was restored by the addition of $Mg^{2+}$, suggesting an important role for this metal during proteolysis. In conclusion, our data suggest that genetic variation in the hPreP gene *PITRM1* may potentially contribute to mitochondrial dysfunctions.



Alzheimer disease (AD) is a progressive neurodegenerative disease associated with short-term memory impairment, spatial disorientation as well as general cognitive decline. The mechanism involved in the accumulation of amyloid β-peptide (Aβ) into extracellular plaques is one major research area. However, recent studies have directed attention toward the intracellular accumulation of Aβ and its presence in mitochondria, reviewed in [1]. These studies suggest that the presence of Aβ in mitochondria induces mitochondrial energy metabolism impairment [5] and an imbalance of mitochondrial fission and fusion [21] contributing to cellular dysfunction in the brain of AD patients. Interestingly, we were able to show that Aβ is transported into mitochondria via the Translocase of the Outer Membrane (TOM) machinery [10].

A few years ago we have identified the mitochondrial peptidasome, PreP, localized in the matrix, which degrades presequences and other short, unstructured peptides [12,15,19]. Importantly, we found human PreP (hPreP) to be responsible for degrading Aβ in mitochondria [8]. PreP belongs to the same pitrilysin oligopeptidase family as Insulin Degrading Enzyme (IDE). In contrast to IDE, hPreP cannot degrade insulin, but does degrade the insulin B-chain. A three-dimensional structural homology model of hPreP based on the 2.1 Å crystal structure of *Arabidopsis thaliana* PreP (*At*PreP) [12] identified two cysteines in close proximity of each other that might form a disulphide bond under oxidizing conditions inhibiting the enzyme, thus making the enzyme sensitive to the oxidative state of its environment. Based on the growing experimental evidence on the presence of and the detrimental effects of Aβ in mitochondria, we believe that hampered degradation of mitochondrial Aβ by hPreP may be an important contributory factor in the etiology of AD.

However, the etiology of AD is complex and mutations in amyloid precursor protein (*APP*; MIM# 104760) [9], presenilin 1 (*PSEN1*; MIM# 104311) [18] and presenilin 2 (*PSEN2*; MIM# 633044) [17] can cause rare dominant early-onset forms of the disease. The vast majority of AD cases is not monogenic and has an age at onset >65 years of age. To date, the ε4 allele of the apolipoprotein E gene (*APOE* ε4; MIM# 107741.0016) is the only confirmed genetic risk factor for late onset AD (LOAD) [6]. In the search

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for additional genetic risk factors, several groups, including our own, have performed association studies on the *IDE* gene and the risk for AD (http://www.alzgene.org). The results from these studies are inconclusive, two studies using Swedish AD cases and controls have reported positive association to variations in *IDE* [4,16]. Interestingly, the overall 3D structures of IDE and PreP are highly similar [11,14]. The homology of the active sites of hPreP and IDE and the results from our functional studies of hPreP [8] urged us to investigate the possible role for the gene encoding hPreP, *PITRM1*, located in chromosomal region 10p15.2, containing 27 exons, and transcribed from the antisense strand as a genetic risk factor for AD. We present a gene-wide association study by genotyping 18 SNPs (including nine tagged SNPs) in *PITRM1* using a total of 673 AD cases and 649 cognitive controls. Moreover, we investigated if there is a functional difference between hPreP alleles. Based on the structural location of the reported non-synonymous SNPs and thereby their potential effect on hPreP proteolysis, four SNPs were selected for further studies of proteolytic activity of different peptide substrates, including Aβ.

The AD cases were recruited at the Memory clinic at the Department of Geriatrics, Karolinska University Hospital, Huddinge, Sweden after informed consent by the subjects or a proxy and the study was approved by the local ethics committee. The cases were: 256 unrelated familial AD (FAD) cases having at least one affected, first-, or second-degree relative; 417 late onset clinical AD (CAD) cases without a positive family history. Cases with mutations in *APP* and *PSEN 1* and *2* were excluded. The National Institute of Neurological and Communicative Disorders and stroke–Alzheimer's Disease and Related Disorders Association criteria [13] were used for the diagnosis.

Two control sample sets were used for the CAD and FAD cases including 348 (SNACK I) and 301 (SNACK II) subjects, respectively (Supplementary Table 1). Controls were randomly selected and frequency matched for sex and age from 3500 individuals included in the ongoing, longitudinal study SNACK (The Swedish National Study on Aging and Care in Kungsholmen [7]; http://www.aldrecentrum.se/snack). All controls had a Mini-Mental State Examination $\geq 28$. DNA was extracted from whole blood according to standard protocols.

Tagged SNPs were selected based on the genotypes from the CEPH European panel in the Hapmap database (*rs7079217*, *rs2306319*, *rs7898695*, *rs4881109*, *rs3752802*, *rs4880597*, *rs9423705*, *rs7900718*, *rs6602033*). The minor allele frequency was >0.20, the minimal coefficient of determination ($r^2$) at which all alleles were to be captured was >0.80 and multi-marker (haplotype) tagging was used [7]. The mean $r^2$ for all common alleles in the CEPH reference panel was 0.94 and the lowest observed $r^2$ was 0.81.

To further cover the coding regions of *PITRM1*, including the location of the active sites [8], we genotyped eight additional coding SNPs (*rs2388560*, *rs3740607*, *rs3765101*, *rs3182535*, *rs4242746*, *rs4609511*, *rs3814596*, *rs12359035*). *Rs12359035*, located in exon 4 where the reported active site is located [8], had a low success rate (85%) in the genotyping process and was thus replaced by *rs 4242748* an intronic SNP, located 53 bp upstream of the active site, and in strong linkage disequilibrium, with *rs12359035* (LD; $D' = 1$ and $r^2 = 1$). *Rs12248937* was genotyped since our functional data suggested a lower proteolytic activity for this SNP. All SNPs are listed in Supplementary Table 2 and available at http://www.Hapmap.org.

The ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, USA) was used for genotyping SNPs *rs4242748* and *rs12248937* and PCRs were performed using the TaqMan Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems, USA). The other 17 SNPs were genotyped at the Mutational Analysis Facility (MAF) Karolinska Institutet, using Sequenom MassARRAY technology (Sequenom Inc., San Diego, CA, USA).

All SNPs were tested for Hardy Weinberg Equilibrium (HWE) in the individual sample sets and in the combined sample set both in cases and controls. Allelic and genotypic associations were investigated as two-sided *P*-values obtained by Chi-squared analysis. For the combined sample set we used logistic regression (LR) analysis in order to correct for potential sampling differences among the two case groups (FAD and CAD) (Supplementary Table 3). SPSS version 14.0 was used for all the above-mentioned statistical analyses. Haploview 3.32 (URL: http://www.broad.mit.edu/mpg/haploview/) was used for estimating linkage disequilibrium (LD) and for the haplotype association analyses (http://www.hapmap.org).

Selected hPreP-SNP variants were generated using the QuickChange Site Directed Mutagenesis Kit (Stratagene) with a pGEX-6P-2 plasmid (Amersham Biosciences) containing mature wt hPreP as template. The corresponding recombinant proteins were overexpressed and purified according to [8,12].

The proteolytic activity of wt hPreP and hPreP-SNP variants was measured using 1 μg purified protein and 1 μg substrate peptide $pF_1\beta$, Aβ(1–40) or Aβ(1–42) in degradation buffer containing 20 mM HEPES-KOH, pH 8.0. The samples were incubated at 37 °C for 5–30 min when using $pF_1\beta$ and for 30 min with Aβ(1–40) and Aβ(1–42). The reaction was stopped by the addition of 2× Laemmli sample buffer, and analyzed on 10–20% Tris–Tricine gels (Bio-Rad) stained with Coomassie Brilliant Blue. Kinetics of proteolysis were monitored using the fluorogenic peptide Substrate V (7-methoxycoumatin-4-yl-acetyl-RPPGFSAFK-2,4-dinitrophenyl, R&D Systems). 1 μg hPreP in degradation buffer was mixed with 1.5 μg Substrate V. The hydrolysis of Substrate V was measured for 120 s using a fluorometer (Fluorolog Jobin Yvon, Horiba group) with excitation and emission wavelengths of 320 nm and 405 nm, respectively. All the assays were performed in the presence or absence of 10 mM $MgCl_2$.

Seventeen SNPs, spanning the whole *PITRM1* gene were analyzed in two Swedish case–control sample sets (Fig. 1). All SNPs were in HWE both in the individual sample sets (FAD and CAD) and in the combined sample series. Using Chi-square analysis, we could not find any genotypic or allelic association between any of the genotyped SNPs in *PITRM1* and AD in the two individual sample sets. Logistic regression was used to exclude sampling differences between the two case groups (CAD and FAD). This was done by adding "case group" as a variable in the analysis. Since this variable was not significant, i.e. there was no significant difference in genotype distribution between the two case groups, we used chi-square analysis for the association testing of the total sample set. No significant allelic or genotypic association was found to any of the genotyped SNPs in the combined sample set. We also tested for haplotype association in the two sample sets, both individually and combined, without detecting significant haplotype association. Chi-square analysis was not possible for SNP *rs12248937* since this SNP was monomorphic (only the G allele was identified in our population) and therefore it was withdrawn. The *P*-values from the different association analysis in this study were all >0.09, and therefore considered non-significant (Supplementary Table 3).

SNPs with identification numbers *rs9423502*, *rs3814596*, *rs12248937* and *rs2279219*, corresponding to mutations in the mature protein L116V, F140S, A525D and I924M, respectively, were selected out of 18 non-synonymous hPreP-SNPs based on their location in the hPreP structural model (Fig. 2A and B). The structural model of hPreP revealed that the protease is arranged in four domains, forming two enzyme halves, connected by a hinge region. Active site residues are located in both halves, which interact to form a large peptidasome chamber [8,12]. Leu-116 is located