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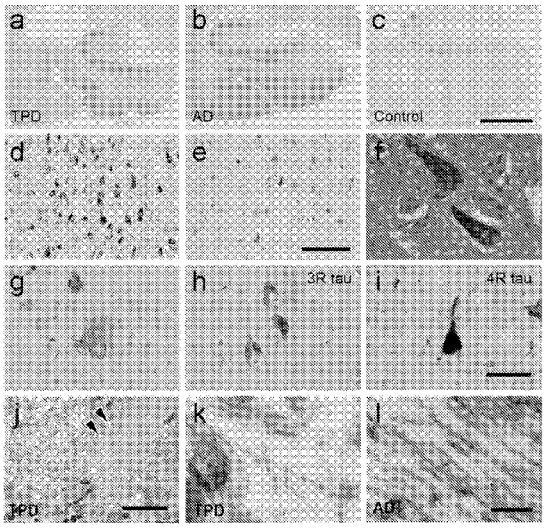
(54) Title: BIOMARKERS FOR TANGLE-PREDOMINANT DEMENTIA

Fig 1



(57) Abstract: This invention relates to the field of screening for, identifying, and diagnosing tangle- predominant dementia (TPD). Specifically, this invention provides various biomarkers for this disease, and methods of using these biomarkers to correctly subclas - sify patients with Alzheimer's-type dementia by differentiating TPD patients from those with classical Alzheimer's disease (AD), as well as prodromal AD, and mild cognitive impairment due to AD.

BIOMARKERS FOR TANGLE-PREDOMINANT DEMENTIA

CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. patent application serial No. 61/662,644 filed June 21, 2012, which is hereby incorporated by reference in its entirety.

FIELD OF INVENTION

This invention relates to the field of screening for, identifying, and diagnosing tangle-predominant dementia (TPD), also known as tangle-only dementia, tangle-predominant senile dementia, senile dementia of the tangle type, senile dementia with tangles, limbic neurofibrillary tangle dementia, Alzheimer's disease with tangles only, as well as other names. Specifically, this invention provides various biomarkers for this disease, and methods of using these biomarkers to correctly subclassify patients with Alzheimer's-type dementia by differentiating TPD patients from those with classical Alzheimer's disease (AD), as well as prodromal AD, and mild cognitive impairment due to AD.

BACKGROUND OF THE INVENTION

The presence of abnormal neuronal and glial filamentous inclusions composed of the microtubule-associated protein tau defines a heterogeneous group of neurodegenerative disorders, termed tauopathies (Dickson (2009)). Alzheimer's disease (AD) is currently classified as a secondary tauopathy, with tangles thought to arise as a result of increased levels of toxic species of the amyloid-beta peptide (A β) (Hardy (2006)). Classically, Alzheimer's disease is associated with amyloid plaques and neurofibrillary tangles (NFT). However, the relationship between amyloid plaques and neurofibrillary degeneration in AD remains unclear. Furthermore, climcopathological studies do not reveal a strong correlation between amyloid plaques and cognitive impairment (Dickson *et al.* (1992); Katzman *et al.* (1998); Terry *et al.* (1991)).

The discovery of mutations in the microtubule-associated protein tau gene *(MAPT)* that cause frontotemporal lobar degeneration (FTLD) show that tau dysfunction on its own is sufficient to induce neurodegeneration (Hutton *et al.* (1998); Gasparini *et al.* (2007)), but the majority of patients with tangles lack such mutations. One example of a sporadic non-mutational primary tauopathy is tangle-predominant dementia (TPD) or tangle-only dementia (TOD) (Bancher and Jellinger (1994); Nelson ***et al.*** (2009); Ulrich *et al.* (1992)). These patients develop neurofibrillary tangles that are regionally, morphologically, ultrastructurally

and biochemically identical to those in moderate-stage AD, yet lack significant Aβ deposition as plaques (Santa-Maria *et al.* (2012)).

TPD patients exhibit neurofibrillary tangles in the medial temporal lobe, progressing to a regional distribution corresponding to moderate-stage AD (Braak stages III-IV) (Braak and Braak (1991)). However, the severity of NFT in TPD more closely resembles end-stage disease (Noda *et al.* (2006)). Currently, neither clinical features nor diagnostic tests can differentiate TPD from early to moderate AD, and the only way to diagnose TPD is post-mortem.

The etiology of TPD is not known. Authors have grouped TPD with frontotemporal lobar degeneration (FTLD) with *MAPT* mutation, but this classification is imperfect given the differences in symptomatology and neuropathological features of FTLD and TPD (Cairns *et al.* (2007); Ikeda *et al.* (1999)).

The scarcity of Aβ deposition that differentiates TPD from classical "plaque and tangle" AD is striking with rigorous histological analysis failing to find more than scattered diffuse plaques or vascular amyloid in a small minority of patients (Ulrich *et al.* (1992); Jellinger and Attems (201 1); Bancher and Jellinger (1994)). Tauopathies are classified neuropathologically using the distribution, morphology and ultrastructure of neurofibrillary tangles, yet no features can differentiate the NFTs in TPD from those in moderate-stage AD. The prevalence of neurofibrillary tangles in normal elderly individuals has prompted suggestions that TPD is a form of pathological or "accelerated" aging (Bouras *et al.* (1994); Junn and Mouradian (2012); Price and Morris (1999); Savva *et al.* (2009)). Finally, TPD may be an AD variant (Jellinger and Bancher (1998)).

The mechanism of how NFTs form in the absence of amyloid plaques is of critical importance. The amyloid cascade hypothesis posits that increased Aβ is the disease trigger in AD, leading to NFT formation and neurodegeneration (Hardy and Selkoe (2002)). The toxic species in AD may be soluble Aβ in the form of pre-fibrillar diffusible assemblies, rather than Aβ deposited in plaques (Walsh and Selkoe (2007)). While the possibility that APP or its catabolites contribute to TPD has not previously been tested experimentally, the consensus criteria for the neuropathological diagnosis of AD require the presence of insoluble Aβ deposited in plaques together with NFT (Hyman *et al.* (2012); Montine *et al.* (2012)).

Currently, there is no way to clinically differentiate TPD from classical plaque and tangle AD, yet the distinction is critical for implementing Aβ-targeted therapies. Given the low Aβ levels in TOD, it is unlikely that Aβ-targeted agents will be useful and they may subject TPD patients to unnecessary risk. Given the predominantly amnestic symptoms, TPD

patients may be clinically classified as having mild cognitive impairment due to AD (Albert *et al.* (201 1)). Unfortunately, the cerebrospinal fluid biomarkers for AD *{i.e.,* low A β and high tau) are predicted to be positive in TPD (Trojanowski *et al.* (2010)). The exception is positron emission tomography (PET)-based amyloid imaging, which may increase recognition of TPD. Notably, as many as one third of amnestic mild cognitive impairment patients are ^{11}C-PIB negative (Devanand *et al.* (2010)), which is compatible with the findings here and by others that TPD is more widespread than acknowledged by the research community. Improved methodology to identify and treat TPD would be highly clinically useful.

Thus, there is a need for methods of screening for, diagnosing or identifying tangle-only or tangle-predominant dementia, particularly differentiating TPD from amyloid driven neurodegeneration *{i.e.,* classical Alzheimer's disease) and other dementing illnesses.

SUMMARY OF THE INVENTION

This invention is based on the surprising discovery that TPD patients, who develop Alzheimer' s-type neurofibrillary tangles that are biochemically identical to those in early to moderate-stage AD, have very low levels of soluble A β. Furthermore, it was discovered that there is non-amyloidogenic APP processing in TPD brain. Genetic analysis demonstrated that TPD is associated with the *MAPT* H1 haplotype in the absence of a coding region mutation, and an additional significant association between TPD and a genomic variation in the *MAPT* 3' UTR, suggesting a novel mechanism whereby post-transcriptional regulation of *MAPT* contributes to tauopathy. These various novel biomarkers can be used to screen for, diagnose, and/or identify TPD in patients who exhibit cognitive impairment, and in particular, to differentiate the disease from AD. Additionally, the knowledge that the variation in the *MAPT* 3'UTR is associated with tauopathy reveals new methods of cellular drug screens.

One embodiment of the present invention is a method for screening, diagnosing, predicting and/or identifying TPD, comprising identifying a subject with cognitive impairment, obtaining biological tissue and/or bodily fluid from the subject, purifying and/or isolating protein from said biological tissue and/or bodily fluid, and detecting the levels of A β protein or peptide in the purified and/or isolated protein sample. The level of A β is compared to the levels in a protein sample from a healthy control and/or a patient known to have AD. If

the levels of Aβ are different, either qualitatively, e.g., by visualization, or quantitatively, *e.g.,* comparison to a known quantity of the protein in a healthy control and/or a subject with AD, the patient can be determined, diagnosed, predicted or identified as having TPD. Specifically, if the level of Aβ in the protein sample from the subject is decreased or lower than the level of Aβ in the protein sample from the healthy control and/or the subject known to have AD, then the patient can be diagnosed or identified as having TPD.

In a preferred embodiment, the patient who is being tested has a cognitive impairment that might be diagnosed as Alzheimer's disease.

The purified and/or isolated protein sample can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The purified and/or isolated protein sample can be obtained from any bodily fluid. Preferred biological fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

The protein can be obtained and processed from the biological tissue or bodily fluid by any method known in the art, in order to obtain a purified and/or isolated protein sample.

Detection of the level of Aβ can be accomplished by any method known in the art, including methods which result in qualitative results, such as ones where the existence of the protein can be visualized, either by the naked eye or by other means, and/or quantitative results. Such methods would include, but are not limited to, quantitative Western blots, immunoblots, quantitative mass spectrometry, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIA), immunoradiometric assays (IRMA), and immunoenzymatic assays (IEMA) and sandwich assays using monoclonal and polyclonal antibodies.

In a preferred embodiment, the results of these methods in the subject are compared to the results of the same method in a healthy control and/or a subject known to have AD.

In a preferred embodiment, the quantity of Aβ is measured in the protein sample from the subject and compared to a reference value of the quantity of Aβ in a healthy control and/or a subject with Alzheimer's disease, wherein the reference value represents a known diagnosis or prediction of AD or normal cognitive function, and finding a deviation in the quantity of Aβ from protein sample of the subject and the reference value, wherein if the quantity of Aβ from protein sample of the subject is decreased or lower than the reference value, then the subject can be determined, diagnosed, predicted or identified as having TPD.

In a preferred embodiment, the level of Aβ42 is detected and/or measured. In another preferred embodiment, the level of 1Aβ40 is detected and/or measured, and in a most preferred embodiment, both the level of Aβ42 and the level of Aβ40 are detected and/or measured.

A further embodiment of the present invention is a method for screening, diagnosing, predicting and/or identifying TPD, comprising obtaining biological tissue and/or bodily fluid from a subject, purifying and/or isolating protein from said biological tissue and/or bodily fluid, and detecting the levels of sAPPa and/or βAPPβ in the purified and/or isolated protein sample. These levels of sAPPa and/or sAPPβ are compared to the levels in a protein sample from a healthy control. If the levels of sAPPa and/or sAPPβ are different, either qualitatively, e.g., by visualization, or quantitatively, e.g., comparison to a known quantity of the proteins in a healthy control, the subject can be diagnosed or identified as having TPD. Specifically, if the level of sAPPa in the protein sample from the subject is increased or higher than the level of sAPPa in the protein sample of the healthy control, then the subject can be diagnosed or identified as having TPD. If the level of sAPPβ in the protein sample from the subject is decreased or lower than the level of βAPPβ in the protein sample from the healthy control, then the subject can be diagnosed or identified as having TPD. While one or the other protein can be tested for, a preferred embodiment is to test for both proteins in the isolated and/or purified protein sample from the subject.

In a preferred embodiment, the subject who is being tested has a cognitive impairment that might be diagnosed as Alzheimer's disease.

The purified and/or isolated protein sample can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The purified and/or isolated protein sample can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

The protein can be obtained and processed from the biological tissue or bodily fluid by any method known in the art, in order to obtain a purified and isolated protein sample.

Detection of the levels of sAPPa and/or βAPPβ can be accomplished by any method known in the art, including methods which result in qualitative results, such as ones where the existence of the protein can be visualized, either by the naked eye or by other means, and/or quantitative results. Such methods would include, but are not limited to, quantitative Western blots, immunoblots, quantitative mass spectrometry, enzyme-linked immunosorbent

assays (ELISAs), radioimmunoassays (RIA), immunoradiometric assays (IRMA), and immunoenzymatic assays (ΓEMA) and sandwich assays using monoclonal and polyclonal antibodies.

In a preferred embodiment, the results of these methods in the subject are compared to the results of the same method in a healthy control.

In a preferred embodiment, the quantity of sAPPa and/or sAPPβ is measured in the protein sample from the subject and compared to a reference value of the quantity of sAPPa and/or sAPPp in a healthy control, wherein the reference value represents a known diagnosis or prediction of normal cognitive function, and finding a deviation in the quantity of sAPPa and/or sAPPβ from protein sample of the subject and the reference value, wherein if the quantity of sAPPa from protein sample of the subject is increased or higher than the reference value, then the subject can be determined, diagnosed, predicted or identified as having TPD, and sAPPβ from protein sample of the subject is decreased or lower than the reference value, then the subject can be determined, diagnosed, predicted or identified as having TPD

Apolipoprotein E (ApoE) alleles, which correlate with AD risk and amyloid plaque load, were found to have different frequencies in patients with TPD. Specifically, a decrease in the ApoE allele, ε4, was seen in patients with TPD as compared to AD, and an increase in alleles, ε2 and ε3, in patients with TPD as compared to patients with AD. Thus, a further embodiment of the present invention is a method for screening, diagnosing, predicting and/or identifying tangle-predominant dementia, comprising obtaining biological tissue and/or bodily fluid from a subject, purifying and/or isolating nucleic acid, including but not limited to cDNA and genomic DNA from the biological tissue and/or bodily fluid, and detecting the presence and/or absence of certain ApoE alleles, including ε2, ε3, and ε4. Specifically, the absence of the ε4 allele in the patient would identify or diagnose the patient as having TPD, and the presence of the ε2 and/or ε3 alleles would identify or diagnose the subject as having TPD. While one of the alleles can be tested for, a preferred embodiment is to test for two, and a preferred embodiment is to test for all three.

In a preferred embodiment, the subject who is being tested has a cognitive impairment that might be diagnosed as Alzheimer's disease.

The purified and isolated nucleic acid can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The purified and isolated nucleic acid can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

The nucleic acid can be purified and isolated using any method known in the art.

Detection of the Apo E alleles can be accomplished by any method known in the art, including, but not limited to, sequencing, hybridization with probes including Southern blot analysis and dot blot analysis, polymerase chain reaction (PCR), PCR with melting curve analysis, PCR with mass spectrometry, fluorescent in situ hybridization, DNA microarrays, single-strand conformation analysis, and restriction length polymorphism analysis.

One preferred method for the detection of the Apo E alleles is to amplify and sequence the Apo E gene and determine if the alleles are present by a comparison to the known sequences for the alleles, ε2, ε3, and ε4.

Detection of the Apoe E alleles can also be accomplished by allele-specific PCR. In this method, primers specific for each Apo E allele are designed from the sequence of the Apo E gene. These primers will anneal to the purified and isolated nucleic acid of the patient only if the particular allele is present.

Another preferred embodiment of this method includes hybridizing the isolated and purified genomic DNA from Apo E allele from the subject with probes comprising the nucleotide sequence of the Apo E alleles. If the probes comprising the nucleotide sequence of Apo E alleles, ε2 and/or ε3 hybridize to the isolated and purified genomic DNA from the subject and/or the probes comprising the nucleotide sequence of ε4 does not hybridize to the isolated and purified genomic DNA from the subject, the subject is determined, diagnosed, predicted or identified as having TPD. In these embodiments, the isolated and purified genomic DNA or the probes must be labeled by methods known in the art for visualization if hybridization occurs.

A further embodiment of the present invention is based upon the surprising findings set forth herein that the haplotype H1 of the *MAPT* iocus, and especially certain single nucleotide polymorphisms (SNP) in the 3'UTR, are highly associated with TPD.

Thus, this embodiment of the present invention is a method for screening, diagnosing and/or identifying tangle-predominant dementia, comprising obtaining biological tissue and/or bodily fluid from a subject, purifying and/or isolating nucleic acid, including, but not limited to, genomic DNA and RNA from the biological tissue and/or fluid, and detecting the presence of the H1 haplotype in the genomic DNA, wherein the presence of the H1 haplotype diagnoses or identifies the patient as having TPD.

In a preferred embodiment, the subject who is being tested has a cognitive impairment that might be diagnosed as Alzheimer's disease.

The purified and/or isolated nucleic acid can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The purified and isolated nucleic acid can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

The nucleic acid can be purified and isolated using any method known in the art.

Detection of the H I haplotype can be accomplished by any method known in the art, including, but not limited to, sequencing, hybridization with probes including Southern blot analysis and dot blot analysis, polymerase chain reaction (PCR), PCR with melting curve analysis, PCR with mass spectrometry, fluorescent in situ hybridization, DNA microarrays, and single-strand conformation analysis.

One preferred method of detection of the H I haplotype is to amplify the *MAPT* locus with primers, sequencing the *MAPT* locus and determining if the H I haplotype is present by a comparison to known sequences of the H I haplotype, such as those found in Table 9. Primers useful in this technique can be manufactured using the sequence of the *MAPT* locus as well as the alleles of the H I haplotype listed in Table 9. The MAPT H*1* and H2 haplotype can be determined by PCR using the Delln9 238 bp marker.

Detection of the H I haplotype can also be accomplished by allele-specific PCR. In this method, primers specific for the H I haplotype are designed from the sequence of the *MAPTH1* haplotype. These primers will anneal to the purified and/or isolated genomic DNA of the patient only if the H I haplotype is present.

Yet another embodiment of the present invention is a method for screening, diagnosing, predicting and/or identifying tangle-predominant dementia, comprising obtaining biological tissue and/or bodily fluid from a subject, purifying and/or isolating the genomic DNA from the tissue or fluid, and detecting the presence or absence of particular polymorphisms found in the 3'UTR of the MAPT gene.

Specifically the presence of the polymorphism designated rs35 134565, with the nucleotide sequence set forth in SEQ ID NO: 1, and/or the absence of the polymorphism designated rs5 820605, with the nucleotide sequence set forth in SEQ ID NO: 2, determines, diagnoses, predicts or identifies a patient as having TPD.

In a preferred embodiment, the subject who is being tested has a cognitive impairment that might be diagnosed as Alzheimer's disease.

The purified and/ or isolated genomic DNA can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The purified and isolated genomic DNA can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

The genomic DNA can be purified and isolated using any method known in the art.

Detection of the polymorphisms can be accomplished by any method known in the art, including, but not limited to, sequencing, hybridization with probes including Southern blot analysis and dot blot analysis, polymerase chain reaction (PCR), PCR with melting curve analysis, PCR with mass spectrometry, fluorescent in situ hybridization, DNA microarrays, single-strand conformation analysis, and restriction length polymorphism analysis.

One preferred method of detection of the polymorphism is to amplify the *MAPT* 3'UTR with primers, sequencing the MAPT 3'UTR and determining if the polymorphisms are present or absent. In a preferred embodiment, the primers with the DNA sequences of SEQ ID NOs: 4 and 5 can be used. The nucleotide sequence obtained of the genomic DNA of the 3'UTR of the *MAPT* gene of the patient is compared to the nucleotide sequences of the polymorphisms. If the DNA from the patient contains the polymorphism designated rs35134565 with the nucleotide sequence of SEQ ID NO: 1 and/or does not contain the polymorphism designated rs5820605, with the nucleotide sequence set forth in SEQ ID NO: 2, the patient is diagnosed or identified as having TPD.

Another preferred embodiment of this method includes hybridizing the isolated and purified genomic DNA from the 3'UTR of the *MAPT* gene from the patient with probes comprising the nucleotide sequence of SEQ ID NOs: 1 and/or 2 and/or nucleotide sequence of the antisense strand of the SEQ ID NOs 1 and/or 2. If the probes comprising the nucleotide sequence of SEQ ID NO: 1 hybridizes to the isolated and purified genomic DNA from the 3'UTR of the *MAPT* gene from the subject and/or the probes comprising the nucleotide sequence of SEQ ID NO: 2 do not hybridize to the isolated and purified genomic DNA from the 3'UTR of the *MAPT* gene from the subject, the subject is diagnosed or identified as having TPD. In these embodiments, the isolated and purified genomic DNA or the probes must be labeled by methods known in the art for visualization if hybridization occurs.

Thus, there are a total of nine biomarkers that can be used to screen for, diagnose and/or identify tangle-predominant dementia or tangle-only dementia. These are:

1. a decreased level of Aβ;
2. an increased level of sAPPa;
3. a decreased level of sAPPβ;
4. the presence of the ApoE allele, ε2;
5. the presence of the ApoE allele, ε3;
6. the absence of the ApoE allele, ε4;
7. the presence of the H1 haplotype;
8. the presence of the polymorphism designated rs35 134565 with the nucleotide sequence comprising SEQ ID NO: 1; and
9. the absence of the polymorphism designated rs5 820605, with the nucleotide sequence comprising SEQ ID NO: 2.

It is contemplated by the present invention that a method that detects any one of these biomarkers can be performed and is sufficient to screen for, diagnose, predict and/or identify tangle-predominant dementia or tangle-only dementia. In a preferred embodiment, methods that detect at least two biomarkers are performed, in a more preferred embodiment, methods that detect at least three biomarkers are performed, in a more preferred embodiment, methods that detect at least four biomarkers are performed, in a more preferred embodiment, methods that detect at least five biomarkers are performed, in a more preferred embodiment, methods that detect at least six biomarkers are performed, in a more preferred embodiment, methods that detect at least seven biomarkers are performed, in a more preferred embodiment, methods that detect at least eight biomarkers are performed, and in the most preferred embodiment, methods that detect all nine biomarkers are performed.

In a further preferred embodiment, a method to detect the H1 haplotype is performed, and if the haplotype is found in the isolated and purified genomic DNA from the patient, the isolated and purified genomic DNA is then analyzed for the presence of the polymorphism designated rs35134565 with the nucleotide sequence of SEQ ID NO: 1; and the absence of the polymorphism designated rs5 820605, with the nucleotide sequence set forth in SEQ ID NO: 2.

BRIEF DESCRIPTION OF THE FIGURES

For the purpose of illustrating the invention, there are depicted in drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 are images of samples from patients with TPD, AD, and controls. Figure 1a depicts immunohistochemical staining of the medial temporal lobe in a TPD patient, with p-tau specific AT8 antisera; Figure 1b depicts immunohistochemical staining of the medial temporal lobe in an AD patient, with p-tau AT8 antisera; and Figure 1c depicts immunohistochemical staining of the medial temporal lobe in a control patient, with p-tau AT8 antisera (scale bar, 7.5 mm). Figures Id and 1e are P-tau immunohistochemical and Bielschowsky silver stains in a TPD patient (CAl), respectively (scale bar, 200 μM). Figures If and 1g are Bielschowsky silver stains in a TPD patient (scale bar, 50 μM). Figures 1h and 1i are immunohistochemical staining with 3R tau (Figure lh) and 4R tau (Figure li) in the hippocampus of a TPD patient (scale bar, 50 μM). Figure lj is ultrastructural analysis of epoxy resin ultrathin sections from CAl of a TPD patient. Arrowheads depict intracellular neurofibrillary tangles (scale bar, 5 μιη). Figures 1k and 11 are high-power imaging of ultrathin sections from CAl of a TPD patient and an AD patient, respectively (scale bar, 200 nm).

Figure 2a is a representative immunoblot using antisera targeting total tau (HT7) and total protein samples from the frontal cortex (BA9) and hippocampus (CAl) demonstrating 69, 64 and 55 kD bands in TPD and control. A high-molecular weight of about 105 kD band is observed in TPD in CAl. Figure 2b is a representative immunoblot using antisera to 3R and 4R tau show similar banding in TPD and controls. Figure 2c are graphs showing the amount of 3R tau, 4R tau and the 3R/4R ratio in control and TPD patients in BA9. Comparisons are by Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Figure 2d is a representative immunoblot with antisera recognizing total tau (HT7) on sarkosyl insoluble tau fractions in AD, TPD and controls. Figure 2e are images from ultrastructural examination of the sarkosyl-insoluble tau fractions in AD and TPD (scale bar, 200 nm).

Figures 3a through 3c show images from immunohistochemical staining with antisera targeting Aβ in the frontal cortex (BA9) reveals Aβ deposition in control and Alzheimer disease (AD), but not TPD (scale bar, 1 mm). Figures 3d through 3f are graphs of ELISA results of Aβ42 levels in BA9 and CAl of control, TPD and AD (Figure 3d); Aβ40 levels in BA9 and CAl of control, TPD and AD (Figure 3e); and the ratio of Aβ42/Aβ40 in BA9 and CAl of control, TPD and AD (Figure 3f). Comparisons are by one-way ANOVA and Tukey's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4a is a representative quantitative immunoblot of extracts from BA9 with APP, sAPPa, and sAPPβ, normalized to GAPDH. Figure 4b is a graph quantifying the results of Figure 4a. Figure 4c is a graph of levels of the APP mRNA in TPD, AD and control. Comparisons are by Student's t-test (b) or one-way ANOVA/Tukey's test (c) or * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5 shows linkage disequilibrium (LD) in *MAPT*. The genotypes for the *MAPT* haplotype tagging SNPs (rsl467967, rs242557, rs3785883, rs2471738, rs9468 and rs7521) together with rs5820605 and rs35134656 from cases and controls («=82) were analyzed in Haploview v4.2 to generate the LD plot. The white horizontal bar represents the chromosomal distance between the polymorphisms. Pairwise D' values are indicated within the diamonds. Variants with strong LD are shown in red. Those without LD are white and variants with uninformative data light blue.

Figure 6 shows the two polymorphisms associated with TPD, rs35 134656 (SEQ ID NO: 1) and rs5820685 (SEQ ID NO: 2).

Figure 7 is a schematic of the dual luciferase reporter system used in Example 7.

Figure 8a are graphs of the total tau mRNA, 3R tau mRNA, 4R tau mRNA, and the ratio of 3R/4R tau mRNA in control, TOD and AD as measured by QPCR. Figure 8b is a graph of luciferase activity versus time in the cell transiently transfected with the three constructs designated, $H1^{3'UTR\text{-}rs58_{20}605}$, $HI^{3'"TM}$, and $H2^{3'UTR}$ and an empty vector. Figure 8c is a graph of luciferase activity in the same cells after application of 300 μM Aβ.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the methods of the invention and how to use them. Moreover, it will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of the other synonyms. The use of examples anywhere in the specification, including examples of any terms discussed herein, is

illustrative only, and in no way limits the scope and meaning of the invention or any exemplified term. Likewise, the invention is not limited to its preferred embodiments.

The terms "tangle-only dementia", "tangle-predominant dementia", "TOD", and "TPD", "tangle-predominant senile dementia", "senile dementia of the tangle type", "senile dementia with tangles", and "limbic neurofibrillary tangle dementia", will be used interchangeably in this application and are defined as patients who develop neurofibrillary tangles that are regionally, morphologically, ultrastructiirally and biochemically similar to those in moderate-stage AD, but lack significant A β deposition as plaques (Santa-Maria *et al.* (2012)).

The term "subject" as used in this application means an animal with an immune system such as avians and mammals. Mammals include canines, felines, rodents, bovine, equines, porcines, ovines, and primates. Avians include, but are not limited to, fowls, songbirds, and raptors. Thus, the invention can be used in veterinary medicine, *e.g.,* to treat companion animals, farm animals, laboratory animals in zoological parks, and animals in the wild. The invention is particularly desirable for human medical applications.

The term "patient" as used in this application means a human subject. In some embodiments of the present invention, the "patient" is one suffering with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase.

The terms "screen" and "screening" and the like as used herein means to test a subject or patient to determine if they have a particular illness or disease, in this case TPD.

The terms "diagnosis", "diagnose", diagnosing" and the like as used herein means to determine what physical disease or illness a subject or patient has, in this case TPD.

The terms "identification", "identify", "identifying" and the like as used herein means to recognize a disease in a subject or patient, in this case TPD.

The terms "prediction", "predict", "predicting" and the like as used herein means to tell in advance based upon special knowledge.

The term "reference value" as used herein means an amount of a quantity of a particular protein or nucleic acid in a sample from a healthy control or a subject known to have AD.

The term "healthy control" would be a human subject who is not suffering from dementing illness and has normal cognitive function. Moreover, it is preferred that the healthy control be age-matched to the subject, within a reasonable range.

The term "mild cognitive impairment" or "MCI" as used in this application means an intermediate stage between the expected cognitive decline of normal aging and the more

serious decline of dementia. It is a brain function syndrome involving the onset and evolution of cognitive impairments beyond those expected based on the age and education of the individual, but which are not significant enough to interfere with their daily activities. It is often found to be a transitional stage between normal aging and dementia. Although MCI can present with a variety of symptoms, when memory loss is the predominant symptom it is termed "amnestic MCI" and is frequently seen as a prodromal stage of Alzheimer's disease.

The terms "Aβ" or "Abeta" are used interchangeably in this application and mean the amyloid beta protein or peptide, derived from the amyloid precursor protein (APP) (Thinakaren and Koo (2008)). Proteolysis of APP by a-secretase or β-secretase produces secreted N-terminal fragments termed sAPPa and sAPPp respectively as well as C-terminal fragments (CTFs). Cleavage by γ-secretase of the β-CTF yields Aβ peptides of predominantly 40 or 42 amino acids. Aβ is the main component of amyloid plaques, deposits found in the brains of patients with Alzheimer's disease.

The terms "*MAPT*", *"MAPT* gene" and *"MAPT* locus" are used interchangeably in this application and mean the microtubule-associated protein tau gene.

The terms "3'UTR" or "3'UTR of the *MAPT* gene" are used interchangeably in this application and mean the critical *cis*-acting regulatory elements that are capable of regulating gene expression on the post-transcriptional level by influencing mRNA stability and localization, among other functions (Aronov *et al.* (2001); Aronov *et al.* (1999)).

As used herein, the term "isolated" and the like means that the referenced material is free of components found in the natural environment in which the material is normally found. In particular, isolated biological material is free of cellular components. In the case of nucleic acid molecules, an isolated nucleic acid includes a PCR product, an isolated mRNA, a cDNA, an isolated genomic DNA, or a restriction fragment. In another embodiment, an isolated nucleic acid is preferably excised from the chromosome in which it may be found. Isolated nucleic acid molecules can be inserted into plasmids, cosmids, artificial chromosomes, and the like. Thus, in a specific embodiment, a recombinant nucleic acid is an isolated nucleic acid. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it is a membrane-associated protein. An isolated material may be, but need not be, purified.

The term "purified" and the like as used herein refers to material that has been isolated under conditions that reduce or eliminate unrelated materials, *i.e.*, contaminants. For example, a purified protein is preferably substantially free of other proteins or nucleic acids with which it is associated in a cell; a purified nucleic acid molecule is preferably

substantially free of proteins or other unrelated nucleic acid molecules with which it can be found within a cell. As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 50% pure; more preferably, at least 90% pure, and more preferably still at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, and other methods known in the art.

The term "antisense DNA" is the non-coding strand complementary to the coding strand in double-stranded DNA.

The term "genomic DNA" as used herein means all DNA from a subject including coding and non-coding DNA, and DNA contained in introns and exons.

The term "nucleic acid hybridization" refers to anti-parallel hydrogen bonding between two single-stranded nucleic acids, in which A pairs with T (or U if an RNA nucleic acid) and C pairs with G. Nucleic acid molecules are "hybridizable" to each other when at least one strand of one nucleic acid molecule can form hydrogen bonds with the complementary bases of another nucleic acid molecule under defined stringency conditions. Stringency of hybridization is determined, *e.g.,* by (i) the temperature at which hybridization and/or washing is performed, and (ii) the ionic strength and (iii) concentration of denaturants such as formamide of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two strands contain substantially complementary sequences. Depending on the stringency of hybridization, however, some degree of mismatches may be tolerated. Under "low stringency" conditions, a greater percentage of mismatches are tolerable *(i.e.,* will not prevent formation of an anti-parallel hybrid).

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression *(e.g.* transcription and translation) of the introduced sequence. Vectors include, but are not limited to, plasmids, phages, and viruses.

Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure

insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular protein or enzyme. Promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes.

The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, used or manipulated in any way, for the production of a substance by the cell, for example, the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays, as described herein.

A "polynucleotide" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA and RNA, and means any chain of two or more nucleotides. A nucleotide sequence typically carries genetic information, including the information used by cellular machinery to make proteins and enzymes. These terms include double or single stranded genomic and cDNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and anti-sense polynucleotide. This includes single- and double-stranded molecules, *i.e.,* DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid

backbone. This also includes nucleic acids containing modified bases, for example thio-uracil, thio-guanine and fluoro-uracil.

The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like. The nucleic acids may also be modified by many means Icnown in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages *{e.g.,* methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages *(e.g.,* phosphorothioates, phosphorodithioates, etc.). Polynucleotides may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators *(e.g.,* acridine, psoralen, etc.), chelators *(e.g.,* metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The polynucleotides may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the polynucleotides herein may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The term "polymorphism" as used herein means the occurrence in the same population of multiple discrete allelic states of which at least two have high frequency (conventionally of 1% or more).

The term "single nucleotide polymorphism" or "small nucleotide polymorphism" as used in this application means a variation in DNA sequence at a single nucleotide.

The terms "percent (%) sequence similarity", "percent (%) sequence identity", and the like, generally refer to the degree of identity or correspondence between different nucleotide sequences of nucleic acid molecules or amino acid sequences of proteins that may or may not share a common evolutionary origin. Sequence identity can be determined using any of a number of publicly available sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, or GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin).

The terms "substantially homologous" or "substantially similar" when at least about 80%, and most preferably at least about 90 or 95%, 96%, 97%, 98%, or 99% of the

nucleotides match over the defined length of the DNA sequences, as determined by sequence comparison algorithms, such as BLAST, FASTA, and DNA Strider. An example of such a sequence is an allelic or species variant of the specific genes of the invention. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system.

The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.,* the limitations of the measurement system, *i.e.,* the degree of precision required for a particular purpose, such as a pharmaceutical formulation. For example, "about" can mean within 1 or more than 1 standard deviations, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term "about" meaning within an acceptable error range for the particular value should be assumed.

Biomarkers for Tangle-Predominant Dementia

TPD is a poorly-understood and under-recognized tauopathy in need of a definitive neuropathological designation. Given the overlapping features with moderate AD and aging, and the absence of reliable markers, recognizing TPD continues to pose a challenge. However, because TPD is A β independent, it is crucial to diagnose TPD correctly, and more specifically differentiate the disease from classical Alzheimer's disease so that the correct therapeutic interventions can be sought. As stated above, the key biomarkers for MCI due to AD are positive or are predicted to be positive *(i.e.,* radiographically identified hippocampal atrophy, memory impairment/medial temporal lobe symptoms, low CSF A β and elevated CSF phospho-tau) in TPD. Thus, improved methodology to identify and treat TPD would be highly clinically useful.

The results set forth herein for the first time provide biochemical and genetic biomarkers for TPD. These biomarkers will allow the correct diagnosis of TPD in a subject exhibiting MCI. Furthermore, these biochemical and genetic biomarkers will allow a

complete understanding of the mechanism of TPD, AD, other taupathies such as Parkinson's disease, as well as neurodegeneration in general.

Moreover, the data reported herein support the argument that TPD is more prevalent than recognized (Example 2), which is consistent with previous reports (Bancher and Jellinger (1994); Ikeda *et al.* (1997); Ulrich *et al.* (1992)). Should estimates of 3-5% of dementia patients prove accurate, TPD may be among the more common neurodegenerative disorders, affecting between 1.1 and 1.8 million individuals globally.

Post-mortem examination of the brains of TPD patients revealed a pattern reminiscent of early to moderate-stage AD. There was gross medial temporal lobe atrophy compared to controls. Unlike most late-stage AD patients, frontal, parietal and occipital cortices are preserved in TPD. Microscopically, TPD brains exhibited severe medial temporal lobe tauopathy with frequent NET, and the NET were immunopositive for 3R and 4R tau as well as for various phospho-tau specific epitopes, which is the same profile as seen in AD and certain rare tauopathies (Ikeda *et al.* (1999); Iseki *et al.* (1997); Noda *et al.* (2006)). Moreover, it was confirmed that extracellular NFT have disproportionate hnmunolabeling for 3R tau in TPD, as previously reported (Iseki *et al.* (2006)). Electron microscopy of the cornu ammonis 1 (CA1) sector of the hippocampal formation showed paired-helical filaments (PHFs) in TPD, as is observed in AD (Kidd (1963)). However, no insoluble amyloid deposits were found in TPD patients (Example 2). Biochemical analysis of tau in both AD and TPD showed little to no differences (Example 3). Taken together, the data show that the NFT in TPD are regionally, histologically, biochemically and ultrastructurally similar to those in early to moderate-stage AD. However, differences between AD and TPD were seen in the biochemical characterization of Aβ and APP. Aβ is derived from the amyloid precursor protein (APP) (Thinakaren and Koo (2008)). Proteolysis of APP by a-secretase or β-secretase produces secreted N-terminal fragments termed sAPPa and sAPPβ respectively as well as C-terminal fragments (CTFs). Cleavage by γ-secretase of the β-CTF yields Aβ peptides of predominantly 40 or 42 amino acids.

There was no significant Aβ deposition in TPD and lower levels of soluble Aβ in TPD brains when compared to AD. The control brains have variable levels of soluble Aβ that overlap with those observed in AD and TPD (Example 4).

Low Aβ may arise from decreased production, decreased fibrillization or increased clearance. Decreased levels of full-length APP in AD as well were observed, consistent with previous reports (Davidisson *et al.* (2001); Wu *et al.* (201 1)). TPD is unlike AD in that BA9

is preserved, leading to the conclusions that low APP levels in TPD reflect differences in underlying APP metabolism rather than neuronal loss and gliosis. Significantly lower sAPPp levels, and significantly higher sAPPa levels, were found in TPD patients, as compared to controls (Example 4).

Finally, there is no difference in the levels of APP mRNA among TPD, AD and control in BA9, suggesting that non-amyloidogenic processing contributes to decreased production of Aβ in TPD (Example 4).

Apolipoprotein E (ApoE) alleles correlate with AD risk and amyloid plaque load (Corder *et al.* (2006); Saunders *et al.* (1993)). The ApoE allele frequency was looked at and there was a significant decrease in ε4 in TPD as compared to age-matched AD and an increase in ε2 and ε3, (Example 4), which is consistent with previous studies (Ikeda *et al.* (1999); Jellinger and Bancher (1998)). These data support the well-established finding that ApoE ε4 is associated with Aβ deposition and ε2 is protective (Corder *et al.* (1993)).

Thus, the discordance in the spatial and temporal development of amyloid plaques and medial temporal lobe NFT suggest that these two pathologies may have distinct pathogenic mediators (Price and Morris (1999)). The findings presented here strongly suggest that toxicity from non-fibrillar soluble Aβ does not contribute to TOD. This conclusion is consistent with the absence of significant amyloid in TOD brain parenchyma when assessed biochemically and immunohistochemically as well as the absence of an association with the ApoE ε4 allele, which is strongly associated with Aβ deposition (Corder *et al.* (1993); Saunders *et al.* (1993)). The observation that TOD patients have an increase in sAPPa provides additional evidence that the amyloidogenic pathway is not dominant in these patients. Increased sAPPa may have a partially protective influence, as sAPPa attenuates excitotoxicity and Aβ-induced tau phosphorylation (Mattson *et al.* (1993); Stein *et al.* (2004)), perhaps serving to delay the onset and severity of TOD relative to AD. In this context, this data are consistent with a role for amyloid in accelerating and amplifying an age-related tauopathy, perhaps by influencing trans-synaptic tau spreading.

Next it was asked whether TPD is associated with changes in the tau gene *{MAPT).* *MAPT* is within an approximately 900 kb ancestral genomic inversion that defines two haplotypes, H1 and H2 (Stefansson *et al.* (2005)). These haplotypes are in complete linkage disequilibrium and do not recombine. Sporadic tauopathies such as progressive supranuclear palsy and corticobasal degeneration as well as Parkinson disease are associated with the H1 haplotype (Baker *et al.* (1999); Bekris *et al.* (2010); DiMaria *et al* (2000)). There are

conflicting reports concerning an association of *MAPT* with AD (Abraham *et al.* (2009); Mukherjee et al. (2007); Myers *et al.* (2005)). How H I confers risk for tauopathy is unclear, but increased expression of 4R tau mRNA isoforms has been implicated (Myers *et al* (2007)), albeit controversially (Hayesmoore *et al.* (2009)). Other factors may play a role. For example, elements in the tau 3' UTR regulate mRNA stability and localization leading to speculation that polymorphisms in this region underlie disease risk (Aronov *et al.* (1999); Aronov *et al.* (1999);Vandrovcova *et al.* (2010)). The approximate 2 Mb H I haplotype is found on chromosome 17 between base pairs 43,000,000 and 45,000,000, and is obtainable in the genome browser at chrl7:43,000,000-45,000,000 (UCSC Genome Browser on Human Feb. 2009 (GRCh37/hgl9) Assembly). The H I haplotype of the *MAPT 3'* UTR is found at chromosome 17 between base pairs 44,101295 and 44,105,727 and is set forth in SEQ ID NO: 6.

The results shown herein confirm that HI is associated with TPD (Example 5).

Additionally, two polymorphisms within the region of *MAPT* encoding the 3' UTR were identified that are significantly associated with TPD, rs5820605 and rs35134656 (Example 6).

3' UTRs are critical cis-acting regulatory elements that are capable of regulating gene expression on the post-transcriptional level by influencing mRNA stability and localization, among other functions (Aronov *et al.* (2001); Aronov *et al.* (1999)). These two distinct polymorphisms, one predicted to confer risk, rs35 134656, found in TPD patients three times more than in controls, and the other protective, rs5820605, are contained within a 10 bp motif with high sequence identity and high conservation, suggesting that the motif is functional. The finding that a single nucleotide deletion in this motif at rs5 820605 suppresses baseline tau 3' UTR activity *in vitro* compared to control H I and H2 3' UTRs suggests that alterations in tau expression underlie tauopathy risk. The increased tau 3' UTR activity that was observed following A β is not impacted by rs5 820605, suggesting that A β responsive elements lie outside this variant (Example 7). The sequences of these polymorphisms, as well as the position of the mutations and conserved 10 bp motif are shown in Figure 6. Together, these finding suggest that dysregulation of complex expression programs may underlie TOD and AD.

The ultimate cause of TPD is unclear at this time. The increased levels of sAPPoc in TPD is a surprising finding. Previous research suggests that sAPPα attenuates excitotoxicity and A β-induced tau phosphorylation, perhaps serving to delay the onset and severity of TPD

relative to AD (Mattson *et al.* (1993)). In this context, the data set forth herein are consistent with a proposed role for Aβ in accelerating and amplifying an age-related tauopathy (Purcell *et al.* (2007)).

Previous work has also suggested a viral etiology for TPD (Nelson *et al.* (2010)),as well as other potential causes of neurofibrillary degeneration, such as mild traumatic injury (Gavett *et al.* (2010)). The regional overlap of NFT between TPD and aging has prompted some investigators to suggest TPD represents "pathological" aging (Jellinger and Bancher (1998)).

Nevertheless, currently, there is no way to clinically differentiate TPD from classical plaque and tangle AD, yet the distinction is critical for implementing Aβ-targeted therapies. Given the low Aβ levels in TPD, it is unlikely that Aβ-targeted agents will be useful and they may subject TOD patients to unnecessaiy risk. Given the predominantly amnestic symptoms, TOD patients may be clinically classified having AD (Albert *et al.* (201 1)).

The data set forth herein for the first time shows biomarkers specific for TPD that can be used to diagnose TPD in a subject clinically classified as MCI. Specifically, TPD can be characterized by:

- Decreased levels of Aβ;
- Increased levels of sAPPα;
- Decreased levels of sAPPβ;
- Increased association of the ApoE allele ε2;
- Increased association of the ApoE allele ε3;
- Decreased association of the ApoE allele ε4;
- Increased association with the H I haplotype;
- Decreased association of the polymorphism rs5 820605 in the 3'UTR of *MAPT;* and
- Increased association of the polymorphism rs35134656 in the 3'UTR of *MAPT.*

Thus, any or all of these characteristics can be used as biomarkers for the screening, the diagnosing, predicting, and/or identifying of TPD, and in particular, distinguishing TPD from AD.

In a preferred embodiment of the methods of screening and diagnosis, a subject with cognitive impairment could be tested for all of the amyloid characteristics, *i.e.,* Aβ peptide, sAPPa peptide, sAPPβ peptide, ApoE allele ε2, ApoE allele ε3 and ApoE allele ε4. A

finding that the subject has decreased levels or low quantities of Aβ peptide, and sAPPβ peptide, along with increased levels or high quantities of sAPPa peptide and the ε2 or ε3 allele, would indicate the subject has TPD.

In another preferred embodiment, a subject with cognitive impairment could be tested for all of the tau characteristics, *i.e.*, the H1 haplotype and the polymorphisms. A finding of the H1 haplotype along with the polymorphism designated rs35234656 would indicate the subject has TPD.

Use of Levels of Aβ as a Screening and Diagnosis Method for TPD

As stated above, and shown in Example 4, TPD is associated with lower levels of Aβ protein or peptide. Thus, one embodiment of the present invention is the screening, diagnosis, prediction or identification of tangle-predominant dementia in a subject, by detection and/or measurement of decreased levels or quantity of Aβ in a sample from a subject with cognitive impairment.

A sample of biological tissue or bodily fluid from a subject with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase, is obtained.

The protein sample can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The protein sample can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

Protein is isolated and/or purified from the sample using any method known in the art including but not limited to the one described in Example 4. Other methods for protein isolation and purification include but are not limited to immunoaffmity chromatography.

Any method known in the art can be used, but preferred methods for detecting and measuring decreased levels of Aβ in a protein sample include quantitative Western blot, immunoblot, quantitative mass spectrometry, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIA), immunoradiometric assays (DRMA), and immunoenzymatic assays (IEMA) and sandwich assays using monoclonal and polyclonal antibodies.

Antibodies are a preferred method of detecting and measuring Aβ in a sample. Such antibodies are available commercially (Covance) or can be made by conventional methods known in the art. Such antibodies can be monoclonal or polyclonal and fragments thereof, and immunologic binding equivalents thereof. The term "antibody" means both a

homologous molecular entity as well as a mixture, such as a serum product made up of several homologous molecular entities.

In a preferred embodiment, such antibodies will immunoprecipitate Aβ peptide from a solution as well as react with Aβ peptide on a Western blot, immunoblot, ELISA, and other assays listed above. In another preferred embodiment, these antibodies will react and detect Aβ peptide in frozen tissue section, say from a brain biopsy.

Antibodies for use in these assays can be labeled covalently or non-covalently with an agent that provides a detectable signal. Any label and conjugation method known in the art can be used. Labels, include but are not limited to, enzymes, fluorescent agents, radiolabels, substrates, inhibitors, cofactors, magnetic particles, and chemiluminescent agents.

The levels or quantity of Aβ peptide found in a sample are compared to the levels or quantity of the peptide in healthy controls and/or patients known to have AD and a deviation in the level or quantity of peptide is looked for. This comparison can be done in many ways. The same assay can be performed simultaneously or consecutively, on a purified and/or isolated protein sample from a healthy control and/or an AD patient, and the results compared qualitatively, *e.g.,* visually, *i.e.,* does the protein sample from the healthy control and/or the AD patient, produce the same intensity of signal as the protein sample from the subject in the same assay, or the results can be compared quantitatively, *e.g.,* a value of the signal for the protein sample from the subject is obtained and compared to a known reference value of the protein in a healthy control and/or the patient with AD.

A lower level or quantity of Aβ peptide in a sample from a subject as compared to the reference value of the level or quantity of Aβ peptide of healthy control and/or a patient known to have AD, would indicate or predict that the subject has TPD.

In a preferred embodiment, the level of Aβ42 is detected or measured in the protein sample by any of the methods set forth above, and compared to the levels or quantity of the reference value of the peptide in healthy controls and/or patients known to have AD. In a further preferred embodiment, the level of Aβ40 is detected or measured in the protein sample by any of the methods set forth above, and compared to the levels or quantity of the reference value of the peptide in healthy controls and/or patients known to have AD. In the most preferred embodiment, the levels of both peptides are detected or measured and a ratio of the level of Aβ42/ Aβ40 is determined. In all these embodiments, if the level or quantity of Aβ42, the level or quantity of Aβ40, and/or the ratio of Aβ42/ Aβ40, is lower than the levels or ratio in healthy controls and/or patients with AD, the subject would be predicted, identified or diagnosed with TPD.

Use of Levels of sAPP as a Screening and Diagnosis Method for TPD

As stated above, and shown in Example 4, TPD is associated with higher levels of sAPPα and lower levels of sAPPp. Thus, one embodiment of the present invention is the screening, diagnosis, prediction or identification of tangle-predominant dementia in a subject, by detection of increased levels or quantities of sAPPa in a sample from a subject with cognitive impairment. Another embodiment of the present invention is the screening, diagnosis, prediction or identification of tangle-predominant dementia in a subject, by the detection of decreased levels or quantities of sAPPp in a sample from a subject with cognitive impairment.

A sample of biological tissue or bodily fluid from a subject with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase, is obtained.

The protein sample can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The protein sample can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plama, saliva, sweat, and urine.

Protein is purified and/or isolated from the sample using any method known in the art including but not limited to the one described in Example 4. Other methods for protein purification and isolation include but are not limited to immunoaffinity chromatography.

Any method known in the art can be used, but preferred methods for detecting increased levels or quantities of sAPPa and/or decreased levels or quantities of sAPPp in a protein sample include quantitative Western blot, immunoblot, quantitative mass spectrometry, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIA), immunoradiometric assays (IRMA), and hnmunoenzymatic assays (lEMA) and sandwich assays using monoclonal and polyclonal antibodies.

Antibodies are a preferred method of detecting sAPPa and/or sAPPp in a sample. Such antibodies are available commercially (American Research Products; Covance) or can be made by conventional methods known in the art. Such antibodies can be monoclonal or polyclonal and fragments thereof, and immunologic binding equivalents thereof. The term "antibody" means both a homologous molecular entity as well as a mixture, such as a serum product made up of several homologous molecular entities.

In a preferred embodiment, such antibodies will immunoprecipitate sAPPa and/or sAPPP peptide from a solution as well as react with sAPPa and/or sAPPp peptide on a

Western blot, or immunoblot, ELISA, and other assays listed above. In another preferred embodiment, these antibodies will react and detect sAPPa and/or sAPPP peptide in frozen tissue section, say from a brain biopsy.

Antibodies for use in these assays can be labeled covalently or non-covalently with an agent that provides a detectable signal. Any label and conjugation method known in the art can be used. Labels, include but are not limited to, enzymes, fluorescent agents, radiolabels, substrates, inhibitors, cofactors, magnetic particles, and chemiluminescent agents.

The levels or quantities of sAPPa and/or sAPPp peptide found in a sample are compared to the levels or quantities of these peptides in healthy controls and a deviation in the level or quantity of peptides is looked for. This comparison can be done in many ways. The same assay can be performed simultaneously or consecutively, on a purified and/or isolated protein sample from a healthy control and the results compared qualitatively, *e.g.,* visually, *i.e.,* does the protein sample from the healthy control produce the same intensity of signal as the protein sample from the subject in the same assay, or the results can be compared quantitatively, *e.g.,* a value of the signal for the protein sample from the subject is obtained and compared to a known reference value of the protein in a healthy control.

Alternatively, as shown in Example 4 and Figure 4a, in a protein sample of a patient with TPD, there was clearly a signal when hybridized with sAPPa antibody, and clearly not a signal when hybridized with sAPPp antibody.

A higher level or quantity of sAPPa and/or the lower level or quantity of sAPPp peptide in a sample from a subject as compared to the reference value of the level or quantity of the peptides in a healthy control would indicate the subject has TPD. In a preferred embodiment, the sample is tested for levels or quantities of both sAPPa and/or sAPPβ peptide.

Use of Apo E Alleles as a Screening and Diagnosis Method for TPD

As stated above and shown in Example 4, TPD is associated with the presence or absence of Apo E alleles. This association can be used to screen for, diagnose or identify TPD.

In order to detect the Apo E alleles associated with tangle-predominant dementia, a biological sample from a subject with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase is obtained and prepared and analyzed for the presence of the Apo E alleles ε2, ε3, and/or ε4. This can be achieved in numerous ways, by a

diagnostic laboratory, and/or a health care provider. Specifically the presence of ε2 and ε3, and/or the absence of ε4 would indicate a diagnosis of TPD.

Any method known in the art can be used to detect the presence or absence of the Apo E alleles. Preferred methods that can be utilized in this analysis are sequencing, hybridization with probes including Southern blot analysis and dot blot analysis, polymerase chain reaction (PCR), PCR with melting curve analysis, PCR with mass spectrometry, fluorescent in situ hybridization, DNA microarrays, single-strand conformation analysis, and restriction length polymorphism analysis.

DNA Encoding the Apo E Alleles

There are three Apo E alleles associated with TPD- ε2, ε3, and ε4.

The sequences of at least the ε2 and ε4 alleles are known. The SNP rs7412 comprises the sequence of the ε2 allele (SEQ ID NO: 7), and SNP rs429358 comprises the sequence of the ε4 allele (SEQ ID NO: 8). Using these two sequences alone is enough to determine which Apo E allele a subject possesses. In other words, if the subject is not positive for the ε2 or ε4 allele, they must possess the ε3 allele.

Such DNA sequences, no matter how obtained, are useful in the methods set forth herein for diagnosing TPD. In the simplest embodiment of the present invention DNA isolated and prepared from a sample of biological tissue and/or bodily fluid from a subject with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase is compared to the known sequences of the Apo E alleles, specifically to the polymorphisms designated rs7412 and rs429358, to screen for, predict, or confirm a diagnosis of TPD.

The isolated DNA can also be used as the basis for probes and primers for used in additional diagnostic procedures for TPD.

Use of H1 Haplotype as a Screening and Diagnosis Method for TPD

As stated above and shown in Example 5, TPD is closely associated with the H1 haplotype of the *MAPT* locus. Thus, a further embodiment of the present invention is the use of this association to screen for, predict, diagnose or identify TPD.

In order to detect the H1 haplotype associated with tangle-predominant dementia, a biological sample from a subject with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase is obtained and prepared and analyzed for the presence of the H1 haplotype, and/or the presence of the H1 subhaplotypes Hlb, Hlc, Hid,

and Hie, as set forth in Table 9. This can be achieved in numerous ways, by a diagnostic laboratory, and/or a health care provider.

Any method known in the art can be used to detect the presence or absence of the H1 haplotype. Preferred methods that can be utilized in this analysis are sequencing, hybridization with probes including Southern blot analysis and dot blot analysis, polymerase chain reaction (PCR), PCR with melting curve analysis, PCR with mass spectrometry, fluorescent in situ hybridization, DNA microarrays, single-strand conformation analysis, and restriction length polymorphism analysis.

DNA Encoding the H1 Haplotype and the H1 Subhaplotypes

One embodiment of the present invention is the use of the isolated DNA encoding the H1 haplotype of the *MAPT gene,* found on chromosome 17 between base pairs 43,000,000 and 45,000,000, and obtainable in the genome browser at chrl7:43,000,000-45,000,000 (UCSC Genome Browser on Human Feb. 2009 (GRCh37/hgl9) Assembly), as a diagnostic for TPD.

Further embodiments of the present invention are methods using the isolated DNA of the H1 subhaplotype alleles set forth in Table 9.

Further embodiments of the present invention are methods of using the isolated DNA of the H1 haplotype of the 3' UTR of the *MAPT gene* comprising the nucleotide sequence of SEQ ID NO: 6.

The present invention also includes the use of the antisense DNA of the H1 haplotype, as well as the DNA sequences listed in Table 9 and SEQ ID NO: 6.

The present invention also includes recombinant constructs comprising the DNA comprising the nucleotide sequence of H1 haplotype of the *MAPT* gene, SEQ ID NO: 6, or the sequences in Table 9 for the H1 subhaplotypes, or the antisense DNA comprising the nucleotide sequence of H1 haplotype of the *MAPT gene,* SEQ ID NO: 6, or the sequences in Table 9 for the H1 subhaplotypes, and a vector, that can be expressed in a transformed host cell. The present invention also includes the host cells transformed with the recombinant construct comprising DNA comprising the nucleotide sequence of H1 haplotype of the *MAPT* gene, SEQ ID NO: 6, or the sequences in Table 9 for the H1 subhaplotypes, or the antisense DNA comprising the nucleotide sequence of H1 haplotype of the *MAPT* gene, SEQ ID NO: 6, or the sequences in Table 9 for the H1 subhaplotypes, and a vector.

Such DNA sequences, no matter how obtained, are useful in the methods set forth herein for diagnosing TPD. In the simplest embodiment of the present invention, DNA isolated and prepared from a sample of biological tissue and/or bodily fluid from a subject

with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase is compared to the DNA sequence of the H1 haplotype of the *MAPT* gene, SEQ ID NO: 6 and/or any of the sequences in Table 9 that correspond to the H1 subhaplotype to confirm a diagnosis of TPD.

The isolated DNA can also be used as the basis for probes and primers for used in additional diagnostic procedures for TPD.

Use of Polymorphisms in the MAPT 3'UTR as a Screening and Diagnosis Method for TPD

As shown by the data herein, there are two polymorphisms within the 3'UTR region of MAPT that are closely associated with TPD. These polymorphisms, designated rs5820605 and rs35 134656, can be used as a diagnosis for TPD. Additionally, there is a conserved 11 bp motif (CAGNCACCCCT) (SEQ ID NO: 3) contained in these two polymorphisms.

In order to detect one of the polymorphisms associated with tangle-predominant dementia, a biological sample from a subject with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase is obtained and prepared and analyzed for the presence of polymorphism rs35 134656, and/or the absence of rs5820605. This can be achieved in numerous ways, by a diagnostic laboratory, and/or a health care provider.

Any method known in the art can be used to detect the presence or absence of the polymorphisms. Preferred methods that can be utilized in this analysis are sequencing, hybridization with probes including Southern blot analysis and dot blot analysis, polymerase chain reaction (PCR), PCR with melting curve analysis, PCR with mass spectrometry, fluorescent in situ hybridization, DNA microarrays, single-strand conformation analysis, and restriction length polymorphism analysis.

DNA Encoding MAPT 3'UTR Polymorphisms

One embodiment of the present invention is the use of the isolated DNA encoding the *MAPT 3'UTR* polymorphism, rs35134656, comprising the nucleotide sequence of SEQ ID NO. 1.

A further embodiment of the present invention is the use of the isolated DNA sequence encoding the *MAPTV* UTR polymorphism, rs5820605, comprising the nucleotide sequence SEQ ID NO: 2.

Another embodiment of the present invention is the use of the isolated conserved 11 base pair DNA from the *MAPT 3'XJTR*, comprising the nucleotide sequence of SEQ ID NO: 3.

The present invention also includes the use of the antisense DNA of SEQ ID NOs: 1, 2, and 3.

The present invention also includes recombinant constructs comprising the DNA having the nucleotide sequence of SEQ ID NOs: 1, 2, and/or 3 or the antisense DNA of SEQ ID NOs: 1, 2, and/or 3, and a vector, that can be expressed in a transformed host cell. The present invention also includes the host cells transformed with the recombinant construct comprising DNA having the nucleotide sequence of SEQ ID NOs: 1, 2, and/or 3 or the antisense DNA of SEQ ID NOs: 1, 2, and/or 3 and a vector.

Such DNA sequences, no matter how obtained, are useful in the methods set forth herein for diagnosing TPD. In the simplest embodiment of the present invention DNA isolated and prepared from a sample of biological tissue or bodily fluid from a subject with ranging from mild to severe, or with pre-dementia in the prodromal phase is compared to the DNA SEQ ID NOs: 1 and/or 2 to confirm a diagnosis of TPD.

The isolated DNA can also be used as the basis for probes and primers for used in additional diagnostic procedures for TPD.

Probes and Primers

Further embodiments of the present invention include probes comprising some or all of the DNA comprising the nucleotide sequence of SEQ ID NOs: 7 and 8, and probes comprising some or all of the DNA with the antisense nucleotide sequence of SEQ ID NOs: 7 and 8. These probes can be used to detect Apo E alleles associated with TPD in a sample of DNA from a subject with cognitive impairment, and confirm a diagnosis of TPD in a subject with cognitive impairment.

Further embodiments of the present invention include probes comprising some or all of the DNA comprising the nucleotide sequence of the H1 haplotype of the *MAPT* locus, SEQ ID NO: 6 and the sequences in Table 9, and probes comprising some or all of the DNA comprising the antisense nucleotide sequence of H1 haplotype of the *MAPT* locus, SEQ ID NO: 6 and the sequences in Table 9. These probes can be used to detect H1 haplotype and subhaplotypes associated with TPD in a sample of DNA from a subject with cognitive impairment, and confirm a diagnosis of TPD in a subject with cognitive impairment.

Further embodiments of the present invention include probes comprising some or all of the DNA comprising the nucleotide sequence of SEQ ID NOs: 1, 2, and 3, and probes comprising some or all of the DNA comprising the antisense nucleotide sequence of SEQ ID

NOs: 1, 2, and 3. These probes can be used to detect the polymorphisms and/or conserved 11 bp motif associated with TPD in a sample of DNA from a subject with cognitive impairment, and confirm a diagnosis of TPD in a subject with cognitive impairment.

Probes contemplated for use in the screening and diagnostic assays of the present invention can be made by any method known in the art, including the procedures outlined below.

In standard nucleic acid hybridization assays, probe must be is labeled in some way, and must be single stranded. Oligonucleotide probes are short (typically 15-50 nucleotides) single-stranded pieces of DNA made by chemical synthesis: mononucleotides are added, one at a time, to a starting mononucleotide, conventionally the 3' end nucleotide, which is bound to a solid support. Generally, oligonucleotide probes are designed with a specific sequence chosen in response to prior information about the target DNA. Oligonucleotide probes are often labeled by incorporating a ^{32}P atom or other labeled group at the 5' end.

Conventional DNA probes are isolated by cell-based DNA cloning or by PCR. In the former case, the starting DNA may range in size from 0.1 kb to hundreds of kilobases in length and is usually (but not always) originally double-stranded. PCR-derived DNA probes have often been less than 10 kb long and are usually, but not always, originally double-stranded. DNA probes are usually labeled by incorporating labeled dNTPs during an *in vitro* DNA synthesis reaction by many different methods including nick-translation, random primed labeling, PCR labeling or end-labeling.

Labels can be radioisotopes such as ^{32}P, ^{33}P, ^{35}S and ^{3}H, which can be detected specifically in solution or, more commonly, within a solid specimen, such as autoradiography. ^{32}P has been used widely in Southern blot hybridization, and dot-blot hybridization.

Nonisotopic labeling systems which use nonradioactive probes can also be used in the current invention. Two types of non-radioactive labeling include direct nonisotopic labeling, such as one involving the incorporation of modified nucleotides containing a fluorophore. The other type is indirect nonisotopic labeling, usually featuring the chemical coupling of a modified reporter molecule to a nucleotide precursor. After incorporation into DNA, the reporter groups can be specifically bound by an affinity molecule, a protein or other ligand which has a very high affinity for the reporter group. Conjugated to the latter is a marker molecule or group which can be detected in a suitable assay. This type of labeling would include biotin-streptavidin and digoxigenin.

Primers for use in the various assays of the present invention are also an embodiment of the present invention. A forward primer for amplifying the full-length *MAPT* 3'UTR having the nucleotide sequence of SEQ ID NO: 4 is one embodiment of the invention, and a reverse primer for amplifying the full-length MAPT 3'UTR having the nucleotide sequence of SEQ ID NO: 5 is yet another embodiment of the present invention.

Additionally other primers useful for the methods of screening and diagnosis of the present invention are also contemplated by the invention and can be prepared by method known in the art as outlined below, using the sequences of the *MAPT* 3'UTR, and H1 haplotype of the *MAPT* gene, as well as the sequences of the Apo E alleles, *e.g.*, polymorphisms rs429358 and rs7412, and the polymorphisms rs5820605 and rs35134656.

The specificity of amplification depends on the extent to which the primers can recognize and bind to sequences other than the intended target DNA sequences. For complex DNA sources, such as total genomic DNA from a mammalian cell, it is often sufficient to design two primers about 20 nucleotides long. This is because the chance of an accidental perfect match elsewhere in the genome for either one of the primers is extremely low, and for both sequences to occur by chance in close proximity in the specified direction is normally exceedingly low. Although conditions are usually chosen to ensure that only strongly matched primer-target duplexes are stable, spurious amplification products can nevertheless be observed. This can happen if one or both chosen primer sequences contain part of a repetitive DNA sequence, and primers are usually designed to avoid matching to known repetitive DNA sequences, including large runs of a single nucleotide

After the primers are added to denatured template DNA, they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat-stable DNA polymerase and DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP), they initiate the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segment, and which will overlap each other.

Screening and Diagnostic Assays

Several methods can be used to screen for, diagnose, predict, and identify TPD in a subject with cognitive impairment utilizing the surprising discoveries of the association of certain Apo E alleles, and the H1 haplotype and the two polymorphisms in the *MAPT* 3'UTR with TPD, as well as a conserved 11 base pair motif in these patients.

The most direct method for screening for and diagnosing TPD in a patient with cognitive impairment ranging from mild to severe, or with pre-dementia in the prodromal phase, is to obtain a sample of biological tissue or bodily fluid from the patient and extracting, isolating and/or purifying the nucleic acid *(e.g.,* genomic DNA, cDNA, RNA) from the tissue or fluid.

The nucleic acid can be obtained from any biological tissue. Preferred biological tissues include, but are not limited to, brain, epidermal, whole blood, and plasma.

The nucleic acid can be obtained from any bodily fluid. Preferred bodily fluids include, but are not limited to, cerebrospinal fluid, plasma, saliva, sweat, and urine.

The nucleic acid is extracted, isolated and purified from the cells of the tissue or fluid by methods known in the art. The nucleic acid, *e.g.,* DNA is then sequenced.

In one embodiment, the nucleic acid is sequenced at the Apo E locus, and the sequenced nucleic acid is then inspected at the Apo E locus for the Apo E alleles. Specifically, the DNA from the patient is compared to the DNA of one or all of the nucleotides comprising the sequences of one of, or both SEQ ID NOs: 7 and 8. The comparison can be made to one sequence, or most preferably both sequences. The presence of the ε2 allele set forth in SEQ ID NO: 7, and/or the absence of the ε4 allele set forth in SEQ ID NO: 8 would indicate the patient has TPD.

In another embodiment, the nucleic acid is sequenced at the *MAPT* locus and the sequenced nucleic acid is inspected at the *MAPT* locus for either the H1 haplotype and/or any of the H1 subhaplotypes. Specifically, the isolated, purified and sequenced DNA from the patient is compared to the DNA with the nucleotide sequences of one or all of the H1 haplotype found on chromosome 17 between base pairs 43,000,000 and 45,000,000, and obtainable in the genome browser at chrl 7:43,000,000-45,000,000 (UCSC Genome Browser on Human Feb. 2009 (GRCh37/hgl9) Assembly), SEQ ID NOs: 6, and the sequences for the H1 subhaplotypes in Table 9. The comparison can be made to one sequence, two sequences, three sequences, four sequences, five sequences, or preferably all six. The presence of any of these DNA sequences in the DNA from the biological tissue or fluid of the subject would indicate the subject has TPD.

Another direct method for screening for or diagnosing TPD in a patient is to inspect the nucleic acid sequenced at the *MAPT* 3'UTR locus for the either of the polymorphisms, and/or the conserved 11 bp motif. Specifically, the isolated, purified and sequenced DNA from the patient is compared to the DNA with the nucleotide sequences of SEQ ID NOs: 1 and/or 2. The comparison can be made to one sequence or both sequences. The presence of

the rs35134565 polymorphism set forth in SEQ ID NO: 1, and/or the absence of the rs5820605 polymorphism set forth in SEQ ID NO: 2 would indicate the subject has TPD.

The DNA from the subject can be sequenced by direct DNA sequencing either manual or automated by methods known in the art such as Sanger sequencing, dideoxy sequencing, and automated fluorescent sequencing.

Screening and diagnostic method of the current invention may involve the amplification of the *MAPT* locus, the 3'UTR of the *MAPT* locus, or the amplification of the Apo E locus. A preferred method for target amplification of nucleic acid sequences is using polymerases, in particular polymerase chain reaction (PCR). PCR or other polymerase-driven amplification methods obtain millions of copies of the relevant nucleic acid sequences which then can be used as substrates for probes or sequenced or used in other assays. An example of PCR is found in Example 7 and primers with the nucleotide sequences SEQ ID NOs: 4 and 5 would be useful in embodiments of the present invention.

Amplification using polymerase chain reaction is particularly useful in the embodiments of the current invention. PCR is a rapid and versatile *in vitro* method for amplifying defined target DNA sequences present within a source of DNA. Usually, the method is designed to permit selective amplification of a specific target DNA sequence(s) within a heterogeneous collection of DNA sequences *(e.g.* total genomic DNA or a complex cDNA population). To permit such selective amplification, some prior DNA sequence information from the target sequences is required. This information is used to design two oligonucleotide primers (amplimers) which are specific for the target sequence and which are often about 15-25 nucleotides long.

Of particular usefulness in the current invention is the use of oligonucleotide primers to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest called allele-specific PCR. These allele-specific primers will anneal only to the alleles of interest. In this case, the primers of the current invention made from the nucleotide sequence of the H I haplotype found on chromosome 17 between base pairs 43,000,000 and 45,000,000, and obtainable in the genome browser at chrl 7:43,000,000-45,000,000 (UCSC Genome Browser on Human Feb. 2009 (GRCh37/hgl9) Assembly), and /or nucleotide sequence set forth in SEQ ID NO: 6 can be used as an initial screen of the genomic DNA from the subject. Only if the DNA contains the H I haplotype of the *MAPT* locus will the primers anneal and amplify the product. Additional primers that target the specific polymorphisms can be designed using the sequence information in SEQ ID NOs: 1 and 2, as well as allele specific primers designed to anneal only to DNA with the particular

polymorphism. This technique can also be used to determine which of the Apo E alleles are present in the Apo E gene of the subject.

Mutation detection using the 5' → 3' exonuclease activity of Taq DNA polymerase (TaqMan™ assay) can also be used as a screening and diagnostic method of the current invention. Such an assay involves hybridization of three primers, the third primer being intended to bind just downstream of one of the conventional primers which should be allele-specific. The additional primer carries a blocking group at the 3' terminal nucleotide so that it cannot prime new DNA synthesis and at its 5' end carries a labeled group. In modern versions of the assay, the label is a fluorogenic group and the third primer also carries a quencher group. If the upstream primer which is bound to the same strand is able to prime successfully, Taq DNA polymerase will extend a new DNA strand until it encounters the third primer in which case its 5' → 3' exonuclease will degrade the primer causing release of separate nucleotides containing the dye and the quencher, and an observable increase in fluorescence.

PCR with melting curve analysis can also be used with the disclosed biomarkers to screen for, identify and diagnose TPD. PCR with melting curve analysis is an extension of PCR where the fluorescence is monitored over time as the temperature changes. Duplexes melt as the temperature increases and the hybridization of both PCR products and probes can be monitored. The temperature-dependent dissociation between two DNA-strands can be measured using a DNA-intercalating fluorophore such as SYBR green, EvaGreen or fluorophore-labelled DNA probes. In the case of SYBR green (which fluoresces 1000-fold more intensely while intercalated in the minor groove of two strands of DNA), the dissociation of the DNA during heating is measurable by the large reduction in fluorescence that results. Alternatively, juxtapositioned probes (one featuring a fluorophore and the other, a suitable quencher) can be used to determine the complementarity of the probe to the target sequence. This technique is sensitive enough to detect single-nucleotide polymorphisms (SNP) and can distinguish between various alleles by virtue of the dissociation patterns produced.

PCR with mass spectrometry uses mass spectrometry to detect the end product. Primer pairs are used and tagged with molecules of known masses, laiown as MassCodes. If DNA from any of the agent of primer panel is present, it will be amplified. Each amplified product will carry its specific Masscodes. The PCR product is then purified to remove unbound primers, dNTPs, enzyme and other impurities. Finally, the purified PCR products are subject of ultraviolet as the chemical bond with nucleic acid and primers are photolabile.

As the Masscodes are liberated from PCR products they are detected with a mass spectrometer.

When a probe is to be used to detect the presence of the Apo E alleles, HI haplotype, the HI subhaplotype alleles, the polymorphisms, rs35234656 and rs5820605 and/or the 11 bp conserved locus, the biological sample that is to be analyzed must be treated to extract the nucleic acids. The nucleic acids to be targeted usually need to be at least partially single-stranded in order to form a hybrid with the probe sequence. It the nucleic acid is single stranded, no denaturation is required. However, if the nucleic acid to be probed is double stranded, denaturation must be performed by any method known in the art.

The nucleic acid to be analyzed and the probe are incubated under conditions which promote stable hybrid formation of the target sequence in the probe and the target sequence in the nucleic acid. The desired stringency of the hybridization will depend on factors such as the uniqueness of the probe in the part of the genome being targeted, and can be altered by washing procedure, temperature, probe length and other conditions known in the art, as set forth in Maniatis *et al.* (1982) and Sambrook *et al.* (1989).

Labeled probes are used to detect the hybrid, or alternatively, the probe is bound to a ligand which labeled either directly or indirectly. Suitable labels and methods for labeling are known in the art, and include biotin, fluorescence, chemiluminescence, enzymes, and radioactivity.

Assays using such probes include Southern blot analysis. In such an assay, a patient sample is obtained, the DNA processed, denatured, separated on an agarose gel, and transferred to a membrane for hybridization with a probe. Following procedures known in the art *{e.g.,* Sambrook *et al.* (1989)), the blots are hybridized with a labeled probe and a positive band indicates the presence of the target sequence. Southern blot hybridization can also be used to screen for the polymorphisms. In this method, the target DNA is digested with one or more restriction endonucleases, size-fractionated by agarose gel electrophoresis, denatured and transferred to a nitrocellulose or nylon membrane for hybridization. Following electrophoresis, the test DNA fragments are denatured in strong alkali. As agarose gels are fragile, and the DNA in them can diffuse within the gel, it is usual to transfer the denatured DNA fragments by blotting on to a durable nitrocellulose or nylon membrane, to which single-stranded DNA binds readily. The individual DNA fragments become immobilized on the membrane at positions which are a faithful record of the size separation achieved by agarose gel electrophoresis. Subsequently, the immobilized single-stranded target DNA sequences are allowed to associate with labeled single-stranded probe DNA. The probe will

bind only to related DNA sequences in the target DNA, and their position on the membrane can be related back to the original gel in order to estimate their size.

Dot-blot hybridization can also be used to screen for the Apo E alleles, H I haplotype and/or polymorphisms. Nucleic acid including genomic DNA, cDNA and RNA is obtained from the subject with suspected TPD, denatured and spotted onto a nitrocellulose or nylon membrane and lowed to dry. The membrane is exposed to a solution of labeled single stranded probe sequences and after allowing sufficient time for probe-target heteroduplexes to form, the probe solution is removed and the membrane washed, dried and exposed to an autoradiographic film. A positive spot is an indication of the target sequence in the DNA of the subject and a no spot an indication of the lack of the target sequence in the DNA of the subject.

A particularly useful application of dot blotting is the use of allele-specific oligonucleotide (ASO) probes. This method distinguishes between alleles that differ by even a single nucleotide substitution. ASO probes are using between 15-20 nucleotides long and are employed under hybridization conditions at which the DNA duplex between the probe and the target are stable only if there is a perfect base complementarity between them.

A further embodiment is the use of ASO reverse dot blotting, wherein an oligonucleotide probe is fixed on a filter or membrane and the target DNA is labeled and provided in a solution. Positive binding of labeled target DNA to a specific oligonucleotide on the membrane is taken to mean that the target DNA has the specific sequence.

DNA microarrays can also be used to screen for the HI haplotype, Apo E alleles and polymorphisms. The surfaces involved are glass rather than porous membranes and similar to reverse dot-blotting, the DNA microarray technologies employ a reverse nucleic acid hybridization approach: the probes consist of unlabeled DNA fixed to a solid support (the arrays of DNA or oligonucleotides) and the target is labeled and in solution.

DNA microarray technology also permits an alternative approach to DNA sequencing by permitting by hybridization of the target DNA to a series of oligonucleotides of known sequence, usually about 7-8 nucleotides long. If the hybridization conditions are specific, it is possible to check which oligonucleotides are positive by hybridization, feed the results into a computer and use a program to look for sequence overlaps in order to establish the required DNA sequence. DNA microarrays have permitted sequencing by hybridization to oligonucleotides on a large scale.

Single strand conformation analysis can also be used to determine if the purified and isolated DNA from a subject has particular allele, haplotype or SNP. The conformation of the

single-stranded DNA can alter based upon a single base change in the sequence, causing the DNA to migrate differently on electrophoresis. The analysis can involve four steps: (1) polymerase chain reaction (PCR) amplification of DNA sequence of interest; (2) denaturation of double-stranded PCR products; (3) cooling of the denatured DNA (single-stranded) to maximize self-annealing; and (4) detection of mobility difference of the single-stranded DNAs by electrophoresis under non-denaturing conditions. Additionally, the SSCP mobility shifts must be visualized which is done by the incorporation of radioisotope labeling, silver staining, fluorescent dye-labeled PCR primers, and more recently, capillary-based electrophoresis.

Kits

It is contemplated that all of the diagnostic and screening assays disclosed herein can be in kit form for use by a health care provider and/or a diagnostic laboratory.

For example, for A β and sAPPa and sAPPp peptide testing, such a kit could include antibodies that recognize the peptide of interest, reagents for isolating and/or purifying protein from a biological tissue or bodily fluid, reagents for performing assays on the isolated and purified protein, instructions for use, and reference values or the means for obtaining reference values for the quantity or level of peptides in a control sample.

In a preferred embodiment, antibodies that recognize A β40, A β42, sAPPa and sAPPp would be included in one kit so that assays for all of the peptides related to amyloid could be performed.

Diagnostic and screening assays based upon nucleotide testing can also be incorporated into kits. For example, probes and/or primers for each of the Apo E alleles, reagents for isolating and purifying nucleic acids from biological tissue or bodily fluid, reagents for performing assays on the isolated and purified nucleic acid, instructions for use, and comparison sequences could be included in a kit for detection of the Apo E alleles. A further embodiment would be a kit with all the components for testing of the Apo E alleles and the peptides related to amyloid.

Kits for screening and diagnosis utilizing the H1 haplotype of the *MAPT* locus are also contemplated by the invention. These kits could include probe and/or primers specific for the H1 haplotype, reagents for isolating and purifying nucleic acids from biological tissue or bodily fluid, reagents for performing assays on the isolated and purified nucleic acid, instructions for use, and comparison sequences could be included in a kit for detection of the H1 haplotype.

Kits for screening and diagnosis utilizing the polymorphisms designated rs35 134565 and rs5 820605 are also contemplated by the invention. These kits could include probe and/or primers specific for the polymorphisms, reagents for isolating and purifying nucleic acids from biological tissue or bodily fluid, reagents for performing assays on the isolated and purified nucleic acid, instructions for use, and comparison sequences could be included in a kit for detection of the polymorphisms.

A preferred embodiment is a kit including components for testing for both the H I haplotype and the polymorphisms.

Drug Screening Assays and Research Tools

All of the biomarkers disclosed herein can be used as the basis for drug screening assays and research tools.

In the simplest models, sAPPa and sAPPp peptide can be used in drug screening assays. The peptides can be used in drug screening tests free in solution or affixed to a solid support. Procaryotic or eukaryotic host cells transformed with nucleotides that express sAPPa or sAPPp peptides can also be used. All of these forms can be used in binding assays to determine if agents being tested form complexes with the peptides.

Thus, a further embodiment of the present invention is a method for screening for drugs comprising contacting an agent to be tested with a sAPPa and/or sAPPp peptide and assaying for the presence of complexes between the peptide and the agent by methods known in the art.

High throughput screening can also be used to screen for drugs. Small peptides or molecules can be synthesized and bound to a surface and contacted with the sAPPa and/or sAPPp peptide and washed. The bound peptide is visualized and detected by methods known in the art.

Antibodies to the peptides can also be used in competitive drug screening assays. The antibodies compete with the agent being tested for binding to the peptides. The antibodies can be used to find agents that have antigenic determinants on the peptides.

The nucleotide markers can also be used in various drug screening assays and as research tools.

Host cells can be transformed with DNA comprising the Apo E alleles, the H I haplotype the *MAPT* gene, the H I haplotype of the 3' UTR, or the polymorphisms designated rs5820605 and rs35234656 by methods known in the art.

The resulting transformed cells can be used for testing for therapeutic agents. Specifically, cells can be transformed with any one of the ApoE alleles, ε2, ε3, or ε4, and contacted with a potential therapeutic agent. The resulting expression of the allele can be detected and compared to the expression of the allele in the cell before contact with the agent.

The expression of the alleles in host cells can be detected and measured by any method known in the art, including but not limited to, luciferase reporter gene assay.

Host cell can also be transformed with the HI 3'UTR as well as the HI 3'UTR with and without the polymorphisms designated rs5820605 and rs35234656. Such a method is exemplified in Example 7 using a luciferase report gene assay. As shown in this Example, these cells can also be contacted with potential therapeutic agents and the expression of the inserted DNA detected and measured before and after contact with the agent.

These transformed host cells can also be used for further research. For instance, as shown in Example 7, these constructs can be contacted with peptides and other substances naturally occurring in both healthy controls and patients with AD and TPD to see the effects on the gene expression.

The HI 3'UTR can also be linked to other cells with measurable phenotypes such as tau. Expression of the gene linked to the HI 3'UTR can be measured before and after the contact with a potential therapeutic agent, as well as a naturally occurring peptide or molecule.

These gene constructs as well as the host cells transformed with these gene constructs can also be the basis for transgenic animals for testing both as research tools and for therapeutic agents. Such animals would include but are not limited to, nude mice and drosophila. Phenotypes can be correlated to the genes and looked at in order to determine the genes effect on the animals as well as the change in phenotype after administration or contact with a potential therapeutic agent.

Examples

The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

Example 1- Patient Samples and Statistical Analysis

Autopsy brain samples were obtained from seven centers (Table 1). The primary source of material was the brain bank at Columbia University Medical Center (CUMC) (New York, NY) (Tables 2 and 3). Secondary sources were the University of California San Diego (San Diego, CA), the University of Kentucky (Lexington, KY), the Banner Health Banner Sun Health Research Institute (Sun City, AZ), Northwestern University (Chicago, IL), the University of Washington (Seattle, WA) and Washington University (St. Louis, MO). Patient data for each component of this study are summarized in Table 4. Neuropathological examination was per the protocols of the respective institutions.

Inclusion criteria for TPD were: 1) frequent NFT corresponding to Braak NPT stage III-IV (Braak *et al.* (1993)) and no or very rare NFT in the frontal, parietal or occipital cortex; 2) no more than sparse amyloid plaques (CERAD (Mirra *et al.* (1991)) score 0 or A); and 3) no other neuropathological substrate for dementia. All TPD cases had been clinically classified pre-mortem as either possible or probable AD (n=31) or mild-cognitive impairment (n=3) by their respective source institutions. For APOE genotype comparisons, neuropathologically-confirmed AD patients aged 75 years or higher from the CUMC cohort categorized as CERAD plaque score of C and Braak NFT stage of V-VI were used. Successful cerebral aging was defined as: 1) age greater than or equal to 80 years; 2) CERAD plaque score of 0; and 3) Braak NFT stage of O-II. All subjects were of Caucasian ancestry.

For the statistical analysis for ELISA, quantitative immunoblotting, luciferase assays, and QPCR experiments, the statistical significance was determined by one-way ANOVA and Tukey test or Student's t-test in GraphPad Prism (GraphPad Software, La Jolla, CA). Statistical outliers and specimens with measurement errors were excluded.

For statistical analysis for APOE comparisons, a Fisher's Exact Test performed in Microsoft Excel was used.

For MAPT haplotype comparisons and single locus associations, a Chi-squared test was performed using plink (Purcell *et al.* (2007).

Table 1- Summary of Patient Data from All Seven Centers

Classification	n	Male	Female	Average Age yr. (Range)	Braak NFT	CERAD Plaque Score	Clin. Diag.
Control	56	23	33	86.9 (67-108)	0-II	0-A	normal
TPD	34	11	23	90.2 (65-103)	III-IV	0-A	AD or MCI

AD	50	19	31	86.7 (73-104)	V-VI	B-C	AD

Table 2- Clinical Patient Data for Patients from CUMC

Study case No.	Age	Sex	Clinical dementia	CDR	Last MMSE	Time since MMSE (yr)
1	65	F	DEM	0.5	22	1
2	76	F	NA	NA	NA	NA
3	80	M	CI	NA	22	5
4	85	M	DEM	1	NA	NA
5	86	F	DEM	NA	29	1
6	86	M	DEM	NA	NA	NA
7	90	F	CI	0.5	NA	NA
8	91	F	DEM	0.5	21	1
9	92	F	DEM	NA	NA	NA
10	93	F	DEM	NA	5	4
11	94	F	DEM	NA	26	1
12	95	F	DEM	1	NA	NA
13	97	F	CI	NA	NA	NA

Table 3- Neuropathological Data of Patients From CUMC

Study case No.	Classification	Braak NFT	CERAD age-related plaque score	PMI (min)
1	TPD	III-IV	0	275
2	TPD	III-IV	0	NA
3	TPD	III-IV	0	286
4	TPD	III-IV	0	1065
5	TPD	III-IV	0	NA
6	TPD	III-IV	0	NA
7	TPD	III-IV	0	325
8	TPD	III-IV	0	117
9	TPD	III-IV	0	705
10	TPD	III-IV	0	120
11	TPD	III-IV	0	280
12	TPD	III-IV	0	110
13	TPD	III-IV	0	205

Table 4- Summary of Patient Data from Each Study

	Control	TPD	AD
Ab ELISA -frontal cortex			
n (male/female)	16 (8/8)	11 (2/9)	8 (4/4)
Average PMI (Standard deviation)	126 (111)	496 (541)	388 (128)
Age at death (range)	86.4 (74 - 94)	83.8 (65 - 103)	85 (73 - 98)
Ab ELISA - hippocampus			
n (male/female)	6 (4/2)	6 (1/5)	5 (2/3)
Average PMI (Standard deviation)	202 (823)	783 (534)	560 (355)
Age at death (range)	79.8 (67 - 92)	92.7 (85 - 103)	91.0 (84 - 98)
APP/sAPP immunoblot - Frontal Cortex			
n (male/female)	11 (5/6)	13 (3/10)	-
Average PMI (Standard deviation)	281 (255)	422 (505)	-
Age at death (range)	87.1 (74 - 97)	86.8 (65 - 96)	-
QPCR			
n (male/female)	9 (4/5)	9 (2/4)	6 (2/4)
Average age at death in yr (range)	87 (55 - 97)	89 (82 - 97)	93 (86 -102)
Average RNA- integrity number	6.1	5.8	4.0
Tau sarkosyl immunoblots - Hippocampus			
n (male/female)	5 (3/2)	6 (2/4)	5 (3/2)
Average PMI in min (standard deviation)	668 (907)	381 (393)	558 (534)
Average age at death in yr (range)	80 (67 - 92)	93 (85 - 103)	91 (84 - 96)
Tau immunoblots - Hippocampus			
n (male/female)	5 (3/2)	5 (0/5)	4 (2/2)
Average PMI in min (standard deviation)	609 (941)	221 (96)	662 (556)
Average age at death in yr (range)	80 (67 - 92)	94 (90-103)	89 (84 - 96)
Tau immunoblots - Frontal cortex			
n (male/female)	12 (6/6)	13 (3/10)	-
Average PMI in min (standard deviation)	326 (321)	422 (505)	-
Average age at death in yr (range)	88 (65 - 94)	86.8 (65 - 96)	-
MAPT resequencing			
n (male/female)	5 (3/2)	10 (9/1)	-
Average age at death in yr (range)	90 (85 - 93)	89 (65 - 97)	-
MAPT association analysis			

n (male/female)	48 (18/30)	34 (11/23)	-
Average age at death in yr (range)	88.1 (78 - 108)	90.2 (82 - 100)	-
APOE association analysis			
n (male/female)	56 (27/29)	32 (10/22)	50 (19/31)
Average age at death in yr (range)	83.3 (80-100)	90.4 (81-100)	86.7 (75-104)

Example 2- Neuropathological Analysis

Materials and Methods

Patient material from Columbia University was subjected to a detailed neuropathological analysis (Tables 2 and 3).

hnmunohistochemistry was performed on 6 μη paraffin-embedded sections as previously described (Crary *et al.* (2006)), using various antisera found in Table 5.

For transmission electron microscopy, a portion of CA1, previously fixed in 10% neutral-buffered formalin, was post-fixed with 2.5% glutaraldehyde in 0.1 M Sorenson's buffer (pH 7.2) followed by 1% Os04 in Sorenson's buffer for one hour and embedded in Lx-1 12 (Ladd Research Industries, Inc.). 60 **nra** sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM- 1200 EXII electron microscope and imaged using an ORCA-HR digital camera (Hamamatsu Photonics, Japan).

Results

A consecutive brain autopsy series of 992 patients performed at Columbia University Medical Center was retrospectively reviewed. Of the 336 patients clinically classified as possible or probable AD (McKhann *et al.* (1984)), 13 meet the neuropathological criteria for TPD (Yamada (2003)). This represents 3.8% of all dementia patients, but increases to 7.3% of patients 90 years or above. There is a female preponderance and an average age of death of 86.9 years (range 65-97 years), which is older than the average for AD patients in this series (74 years).

Post-mortem examination of these 13 TPD patients reveals a pattern reminiscent of early to moderate-stage AD. There is gross medial temporal lobe atrophy compared to controls. Unlike most late-stage AD patients, frontal, parietal and occipital cortices are

preserved in TPD. Microscopically, TPD brains exhibit severe medial temporal lobe tauopathy with frequent NFT (Figures 1a, ld-g). All these cases exhibit numerous extracellular ("ghost") tangles (Figure lg), associated with abnormal degenerating argyrophilic neurites.

Consistent with previous studies, NFT in TPD are immunopositive with specific antisera to 3R and 4R tau (Figures 1h and i), as well as for various phospho-tau specific epitopes shown in Table 6, which is the same profile as seen in AD and certain rare tauopathies (Ikeda *et al*.(1999); Iseld *et al.* (1997); Noda *et al.* (2006)). Moreover, it was confirmed that extracellular NFT have disproportionate immunolabeling for 3R tau in TPD, as previously reported (Iseki *et al.* (2006)).

To determine whether there are unique ultrastructural features in TPD, electron microscopy was performed in the cornu ammonis 1 (CA1) sector of the hippocampal formation. Examination of epoxy resin ultrathin sections shows filaments that are suggestive of paired-helical filaments (PHFs) in TPD (n=4), as is observed in AD (Figures lj-1) (Kidd (1963)). Ultrathin sections were also evaluated for insoluble amyloid deposits and inclusions, but none were observed. Aside from NFT, there are no ubiquitin-positive inclusions and no more than incidental a-synuclein-positive inclusions in the locus coeruleus, pars compacta of the substantia nigra, hypothalamus and substantia innominata in two patients. Vascular disease is frequent, as is common in aging, but there was no ischemic injury sufficient in magnitude or distribution, to cause dementia.

Table 5- Antisera				-	-
Antigen (clone)	**Dilution**	**Type**	**Epitope**	**Notes**	**Source**
APP (22C1 1)	1:4000	Mouse Monoclonal	N-term	Detects full-length and secretory fragments	Millipore
APP/A β (6E10)	1:1000	Mouse Monoclonal	AA 1-16 of b-amyloid	Detects APP and Ab	Covance
sAPPa (2B3)	1:1000	Mouse Monoclonal	Neo-epitope after a-secretase cleavage	No cross reactivity with full-length APP	American Research Products
sAPPb	1:500	Rabbit Polyclonal	Neo-epitope after b-secretase cleavage	No cross reactivity with full-length APP	Covance
Tau (HT7)	1:1000	Mouse Monoclonal	-	Total tau	Thermo Scientific
Tau (RD3, 8E6/C1 1)	1:1000	Mouse Monoclonal	209-224	3R specific	Millipore
Tau (RD4, 1E1/A6)	1:500	Mouse Monoclonal	275-291	4R specific	Millipore
Tau (AT8)	1:500	Mouse Monoclonal	S202/T205	Late epitope	Thermo Scientific
Tau (MC6)	1:500	Mouse Monoclonal	S235	-	Dr. Peter Davies
Tau (CP9)	1:500	Mouse Monoclonal	T231	Affects MT binding, early epitope	Dr. Peter Davies
Tau (TG3)	1:500	Mouse Monoclonal	T231	Conformation specific	Dr. Peter Davies
Tau (PG5)	1:500	Mouse Monoclonal	S409	-	Dr. Peter Davies
Tau (CP13)	1:500	Mouse Monoclonal	S202	-	Dr. Peter Davies
Tau (CP3)	1:500	Mouse Monoclonal	S214	-	Dr. Peter Davies
GAPDH	1:2500	Mouse Monoclonal	-	-	Millipore
Ubitquitin	1:300	Mouse Monoclonal	-	-	DakoCytomation
a-synuclein (UKKM51)	1:50	Mouse Monoclonal	-	-	Leica
TDP-43	1:2000	Mouse Monoclonal	-	-	ProteinTech Group

Table 6- Analysis of phospho-tau in paraffin sections from TOD by immunohistochemistry														
	Hippocampus (CA1)			Amygdala			Temporal cortex (BA38)			Frontal cortex (BA9)		
Antisera	TPD	AD	Control	TPD	AD	Control	TPD	AD	Control	TPD	AD	Control
AT8 (pS202/pT205)	+	+	-	+	+	+/-	+	+	+/-	-	+	-
MC6 (pS235)	+	+	+/-	+	+	+/-	+	+	+/-	-	+	-
CP9 (pT231)	+	+	+/-	+	+	+/-	+	+	+/-	-	+	-
TG3 (pT231)	+	+	+/-	+	+	+/-	+	+	+/-	-	+	-
PG5 (pS409)	+	+	+/-	+	+	+/-	+	+	+/-	-	+	-
CP13 (pS202)	+	+	+/-	+	+	+/-	+	+	+/-	-	+	-
CP3 (pS214)	+	+	+/-	+	+	+/-	+	+	+/-	-	+	-
+ = frequent +/- = scattered, - = negative												

Example 3- Biochemical Analysis of Tau

Materials and Methods

For biochemical analysis of tau, protein extracts were prepared from the Brodmann area 9 (BA9) of the frontal lobe, and CA1, of fresh-frozen human brain from patients in the expanded cohort described in Example 1 as described by Takahashi *et al.* (2002) with modifications. Briefly, fresh-frozen brain tissue was homogenized using 10 volumes (wt/vol) of extraction buffer containing 20 mM (4-[2-hydroxyethyl]-l-piperazineethanesulfonic acid]) (HEPES), pH 7.4, 100 mMNaCl, 20 mMNaF, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM EDTA containing a Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) supplemented with 2 mM PMSF using 15 strokes with a Teflon-coated pestle. Homogenates were centrifuged at 3000 x g at 4 °C for 5 minutes. The supernatant (crude total tau fraction) was aliquoted and stored at -80 °C.

Preparation of sarkosyl-soluble and insoluble fractions was carried out as described Ksiezak-Ridings and Wall (1994). Briefly, homogenates were centrifuged at 27,000 x g for 20 minutes. The pellet was resuspended in buffer containing 0.8 M NaCl and 10% sucrose at 10 ml/g of initial tissue and recentrifuged at 27,000 x g for 20 minutes. The supernatant was incubated in the presence of 1% sarkosyl for 1 hour at 25 °C. Finally, the sample was centrifuged at 100,000 x g for 2 hours. The resulting pellet was considered the sarkosyl-insoluble tau fraction.

Samples were resolved by 10% SDS-PAGE, and analyzed by immunoblot with various antisera (Table 5). Some sarkosyl-insoluble fractions were applied to grids, allowed to dry for 20 minutes and negatively stained with 1% lithium phosphotungstate and examined by electron microscopy in Example 2.

Results

To determine whether differences in tau isoform expression occur in TPD, a biochemical analysis was performed in the Brodmann area 9 (BA9) region of the frontal cortex and CA1 region of the hippocampus using an expanded cohort that included specimens from multiple AD research centers in the United States. In both TPD (n=13) and control (n=T2) subjects, immunoblots using total protein from BA9 and probed for tau reveal the three major bands at 55, 64 and 69 kD (Figure 2a). In CA1 of the TPD patient, an additional high-molecular weight band is present at about 105 kD, representing tau aggregates.

Immunoblots using antisera specifically recognizing 3R and 4R tau show no difference in the levels or ratio of tau isoforms between TPD (n=13) and control (n=l 1; Figures 2b and 2c). Protein fractions enriched for insoluble tau isolated by sarkosyl extraction from CAl and examined by immunoblot reveal no difference in the tau isoform expression in TPD (n=5) and in AD (n=6) (Figure 2d).

Ultrastructural studies of sarkosyl fractions confirm the presence of PHFs in TPD as found in AD (Figure 2e).

Taken together, the data show that the NFTs in TPD are regionally, histologically, biochemically and ultrastructurally similar to those in early to moderate-stage AD.

Example 4- Biochemical Characterization of A β and APP in TPD

Materials and Methods

For biochemical analysis of A β, APP and sAPPa/ β, preparation of protein extracts was performed as described by Schmidt *et al.* (2005) with modifications. Fresh-frozen autopsy brain tissue from patients in Example 1, was homogenized with 10 volumes (wt/vol) of buffer [250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA)] containing a Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) using 20 strokes with a Teflon-coated pestle, aliquoted and frozen at -80°C. Protein concentration was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL). For extraction of soluble A β, homogenate was combined with an equal volume of 0.4% diethylamine (DEA) in 100 mM NaCl and centrifuged at 100,000 x g for 1 hr at 4 °C. Then, the supernatant was combined with an equal volume of 0.5 M Tris base, pH 6.8.

ELISAs were performed using A β x-40 and x-42 BetaMark chemiluminescent kits (Covance Inc., Princeton, NJ).

For immunoblotting, the homogenates were separated into membrane (pellet) and cytosolic (supernatant) fractions by centrifugation at 100,000 x g for 1 hour at 4°C. The pellet was resuspended in homogenization buffer, subjected to SDS-PAGE, transferred to nitrocellulose and probed with various antisera (Table 5) and visualized by chemiluminescence. Densitometric analysis was performed using NIH Image J.

Results

While, immunohistochemistry shows no significant Aβ deposition in TPD (Figures 3a-c), soluble Aβ is histologically invisible. ELISAs show significantly lower soluble Aβ42 levels in TPD («=11, $p < 0.001$) and controls ($n=\6, p < 0.05$) as compared to AD («=8) in BA9 (Fig. 3d). ELISAs also show significantly lower levels of soluble Aβ42 in TPD ($n=6, p < 0.001$) and controls ($n=5, p < 0.001$) compared to AD (n=5) in CA1, a region undergoing neurodegeneration in TPD (Fig. 3d). Significant soluble Aβ42 levels are lower in TPD when compared to control subjects in BA9. Measurements of the less fibrillogenic Aβ40 species (Jarrett *et al.* (1993)) reveal significantly lower soluble Aβ40 levels in TPD ($p < 0.001$) and control ($p < 0.01$) than AD in BA9 but not CA1 (Fig. 3e). There is a higher Aβ42/40 ratio in AD in CA1 compared to TPD ($p < 0.001$) and control ($p < 0.001$), but there is no difference between TPD and control (Fig. 3f).

These findings show that TPD brain parenchyma has low levels of soluble Aβ when compared to AD. The control brains have variable levels of soluble Aβ that overlap with those observed in AD and TPD.

Low Aβ may arise from decreased production, decreased fibrillization or increased clearance. Quantitative immunoblots reveal a significantly reduced level of the full length APP holoprotein in TPD («=14) compared to controls ($n=l\,l, p < 0.001$) in BA9 (Figures 4a and b). Decreased levels of full-length APP in AD as well were observed, consistent with previous reports (Davidisson *et al.* (2001); Wu *et al.* (201 1)). TPD is unlike AD in that BA9 is preserved, leading to the conclusions that low APP levels in TPD reflect differences in underlying APP metabolism rather than neuronal loss and gliosis.

Using specific antisera recognizing neoepitopes formed by secretase cleavage, significantly lower ≤APPβ levels were found ($p < 0.001$), and significantly higher sAPPa ($p < 0.05$) (n=14), as compared to controls («=11) in BA9.

Finally, there is no difference in the levels of APP mRNA among TPD («=8), AD ($n=6$) and control (n=9) in BA9 (Figure 4c), suggesting that non-amyloidogenic processing contributes to decreased production of Aβ in TPD.

Apolipoprotein E (ApoE) alleles correlate with AD risk and amyloid plaque load (Corder *et al.* (2006); Saunders *et al.* (1993)). The ApoE allele frequency was determined and found both a significant decrease in ε4 (p = 5.78 x 10-7) in TPD (n=32) compared to age-matched AD (n=50) and an increase in ε2 (p = 5.58 x 10-5) and ε3 (p = 0.037), (Table 7), which is consistent with previous studies (Ikeda *et* a/.(1999); Jellinger and Bancher (1998)). Compared to controls (n=56), there are decreased ε4 and increased ε2 frequencies in TPD,

but these differences are not significant. These data support the well-established finding that ApoE ε4 is associated with Aβ deposition and ε2 is protective (Corder *et al.* (1993)).

Table 7- *APOE* Genotypes

		Allele count		*p* v TPD (e2)		*p* v TPD (e3)		*p* v TPD (e4)	
	e2 (%)	e3 (%)	e4 (%)	one-tailed	two-tailed	one-tailed	two-tailed	one-tailed	two-tailed
TPD	12 (19)	49 (77)	3 (4)						
Control	12 (11)	82 (77)	14 (13)	0.12	0.18	0.54	1.00	0.063	0.112
AD	1 (1)	62 (62)	37 (37)	**5.58E-05**	**5.58E-05**	**0.037**	0.061	**5.78E-07**	**1.43E-06**

significant p values are in bold (Fischer's exact test)

Example 5- Genetic Analysis of MAPT in TPD

Materials and Methods

Resequencing and association analysis

For genomic DNA isolation, fresh-frozen brain was lysed overnight at 55°C under continuous rotation in 500 µï of buffer [4 M urea, 10 mM of EDTA, 0.5% sarkosyl, 0.1 M Tris HC1 pH (8.0), 0.1 M NaCl and 20 mg/ml proteinase K]. DNA was purified by phenol-chloroform extraction. *MAPT* target enrichment was performed with the RDT1000 system (RainDance Technologies, Lexington MA) using 464 primer pairs spanning greater than 99.9% of MAPT totaling 140,252 bp (hgl8; chrl7: 41,324,942 - 41,465,194) and 243,995 bp of amplicons designed using Primer3 software. Amplicons were sequenced on the 454 platform (Roche 454 Life Sciences, Branford, CT). Performance was analyzed using CLC Genomics Workbench (CLC bio, Cambridge, MA; Table 8).

Variants were identified with gsMapper (Roche). Variants were called if identified on three or more reads, with a total read coverage of six, and counted only if they were observed on forward and reverse reads. Variants on 10- 90% of reads were called as heterozygous and those on greater than 90% homozygous. Allele counts and frequencies were calculated and used to generate p values (Fisher's exact test). Variants meeting one the following criteria (after filtering for H2 haplotype-tagging variants) were included for validation: 1) in coding regions or untranslated regions; 2) TPD specific and control specific; 3) $p < 0.025$ (Fisher's exact test, allelic, unadjusted). Validation and genotyping of variants was performed on a

Sequenom MassArray iPlex platform (Sequenom, San Diego, CA) or Sanger sequencing. Subjects were also genotyped for APOE status using the rs7412 and rs429358 polymorphisms (Ghebranious *et al.* (2005); Clark *et al.* (2009)).

Results

Using a set of markers previously employed to tag MAPT haplotype diversity (Pittman *et al.* (2005)), there is no observed difference in the common H1 subhaplotypes, including Hlc, between TPD (n=34) and controls (n=48). However, there is a difference in the H2 frequency in TPD compared to controls ($p = 0.015$) (Table 9), but this finding is not significant following Bonferroni correction ($p = 0.075$).

Post-hoc analysis reveals that the trend towards significance is derived from differences between TPD patients and a subset of controls. Neurodegeneration is common in elderly individuals classified as cognitively normal (Bouros *et al.* (1994); Price and Morris (1999)). Employing a model proposed by Rowe and Kahn (1987), a subset of the oldest-old controls was identified (average age = 89.3 years) that are exceptional based on their mild NFT burden and absence of amyloid plaques. This control group, termed "successful cerebral aging", represented the ultimate in healthy brain aging. When successful cerebral aging controls (n=28) are compared directly to TPD (Table 10), the difference in H1 allele frequency is highly significant ($p = 0.004$), even after adjusting for multiple comparisons ($p = 0.022$). These results are consistent with the hypothesis that the H1 haplotype is a risk factor for limbic NFT formation in TPD and that H2 is protective.

Table 8- *MAPT* resequencing summary statistics										
Sampie ID	**Classification**	**Mapped Reads**	**Target Reads**	**Specificity**	**Mean Base Coverage**	**CI**	**CIO**	**C20**	**CIOO**	**Base coverage**
1	Control	33,448	26,763	80.0%	68	99.4%	95.6%	87.9%	20.8%	93.3%
2	Control	49,530	30,143	60.9%	78	99.5%	96.4%	90.3%	26.1%	93.6%
3	Control	52,629	46,781	88.9%	132	99.3%	98.5%	96.9%	61.8%	95.6%
4	Control	53,118	33,749	63.5%	88	99.5%	97.1%	91.7%	33.2%	93.6%
5	Control	81,592	66,067	81.0%	186	99.5%	98.9%	98.1%	78.3%	95.4%
6	TOD	77,646	60,200	77.5%	166	99.5%	99.1%	98.1%	74.4%	96.1%
7	TOD	121,761	66,311	54.5%	161	99.6%	98.4%	96.6%	64.1%	92.7%
8	TOD	48,950	37,029	75.6%	98	99.5%	97.2%	92.4%	40.0%	93.0%
9	TOD	62,833	31,373	49.9%	83	99.5%	97.4%	92.2%	31.9%	94.6%
10	TOD	27,213	18,812	69.1%	50	99.1%	93.5%	82.6%	5.8%	94.6%
11	TOD	48,171	27,617	57.3%	77	99.5%	95.8%	90.3%	28.4%	93.1%
12	TOD	71,422	55,360	77.5%	152	99.6%	98.9%	97.5%	70.1%	95.6%
13	TOD	45,344	29,557	65.2%	76	99.4%	95.3%	88.3%	25.3%	91.7%
14	TOD	46,755	31,207	66.7%	76	99.4%	94.7%	88.5%	25.8%	91.3%

15	TOD	80,843	60,510	74.8 %	149	99.5 %	98.6 %	96.8 %	61.9 %	93.8 %
	Average	60,084	41,432	69.5 %	109	99.5 %	97.0 %	92.5 %	43.2 %	93.9 %

TOD = tangle-only dementia; Mapped reads: total number of reads mapping to the human genome; target reads: mapped reads that include the target; mean base coverage: average base coverage within target. The target includes all amplicon sequences, with primer sequences excluded. CI: % of target that has at least 1x base coverage. Note, non-unique sequencing reads are mapped randomly. C20: % of target that has at least 20x base coverage. CI00: % of target that has at least lOOx base coverage. Base coverage (0.2x of mean): % of target that has at least 20% of mean base coverage.

Table 9- Association of common *MAPT* haplotypes with TPD v. Control

		TPD (n=34)	**v. Control (n=48)**			
Haplotype	**Allele**	**Frequency**	**Frequency**	**CHISQ**	**P**	**Corrected**
H2a	AGGCCG	0.21	0.39	5.921	0.0015	0.075
H1b	GGGCTA	0.12	0.1	0.165	0.685	1
H1c	AAGTTG	0.16	0.09	1.453	0.228	1
H1d	AAGCTA	0.04	0.05	0.195	0.659	1
H1e	AGGCTA	0.15	0.1	1.193	0.275	1

Table 10- Association of common *MAPT* haplotypes with TPD v. Successful Cerebral Aging

		TPD (n=34)	v. Successful Cerebral Aging (n=28)			
Haplotype	**Allele**	**Frequency**	**Frequency**	**CHISQ**	**P**	**Corrected**
H2a	AGGCCG	0.21	0.45	8.077	0.004	0.022
H1b	GGGCTA	0.12	0.09	0.356	0.551	1
H1c	AAGTTG	0.16	0.11	0.588	0.443	1
H1d	AAGCTA	0.04	0.06	0.212	0.646	1
H1e	AGGCTA	0.15	0.07	1.865	0.172	0.86

Haplotype IDs based on Pittman *et al.* (2005) (rsl467967, rs242557, rs3785883, rs2471738, rs9468, and rs7521, Chi-squared test (X2), significant values after adjustment for multiple testing in bold

Example 6-TPD is Associated with a Variation in the MAPT 3'TJTR

Materials and Methods

To identify genetic variation that may be associated with TPD risk, MAPT was resequenced as described in Example 5 in a cohort of ten TPD patients and five successful cerebral aging controls. Resequencing was performed using multiplex PCR for target enrichment followed by large-scale parallel pyrosequencing of amplicons (Table 8) The target region is 140 kb in total length and completely contained within the ancestral inversion, encompassing all of MAPT, including the promoter (which overlaps with LOC100128977), introns, exons and untranslated regions, as well as the saitohin gene (STH), LOC100130148, and approximately 2 kb of KIAA1267.

Results

Using this approach, a mean base coverage of 109 reads and an average completeness of 99.5% was obtained. Sequence reads were then mapped back to the human genome project reference sequence, and 1236 variants in total were identified, 705 of which are found in dbSNP and the remaining 531 were novel.

15 variants were found to be within MAPT coding regions, 13 of which were known (rsl0445337, rsl052551, rsl052553, rsl 1568305, rsl7651549, rsl7652121, rs2258689, rs62063786, rs62063787, rs62063845, rs63750072, rs63750222, rs63750417). Of these variants, 11 reside in exons that are not expressed in the central nervous system *{i.e.*, exons 4a, 6 and 8) (Andreadis (2005)). The remaining four coding region variants in exons 7 and 9

are synonymous. Consistent with previous reports, much of the variation is derived from differences between H1 and H2, which are in complete linkage disequilibrium (Stefansson *et al.* (2005)).

Next, an association analysis was performed testing 20 variants identified by resequencing (2 coding and 18 noncoding). Variants were selected for validation and analysis based on their frequency, genomic location and p value. The two most significant associations (rs5 820605; p = 0.032) and (rs35134656; p = 0.015), identified when the TPD patients (n=34) are compared to all controls (n=48) do not survive strict Bonferroni correction for multiple testing (Table 10). However, when TPD is directly compared to successful cerebral aging controls (n=28), the differences in these two variants are highly significant, with both maintaining significance following correction: rs5820605 (p = 0.002, OR 0.32, CI95 = 0.15 - 0.67 and rs35 134656 (p = 0.002, OR 4.76, CI = 1.66 - 13.66). The frequency of rs5820605 is 0.33 in TPD, but increases to 0.61 in successful cerebral aging, suggesting that it may be protective. In contrast, rs35 134656 has a frequency of 0.09 in the controls, but increases to 0.32 in TPD, suggesting that it is a risk allele.

It was determined that rs5820605 and rs35134656 are in linkage disequilibrium (LD) with the H2-tagging SNP rs9486, showing that both these variants are on the H1 background (Figure 5). In fact, both variants are situated within the MAPT 3'UTR. Both of these variants are insertion-deletion polymorphisms. The two variants are 1246 base pairs from each other, but are not in linkage disequilibrium. Instead, sequence alignment reveals two instances of a conserved 11 bp motif (CAGNCACCCCT) (SEQ ID NO: 3) containing these two polymorphisms (Figure 6). In both cases, the TPD-associated form of the polymorphisms are predicted to disrupt this consensus, suggesting that rs5820605 and rs35134656 are contained within functional elements in the MAPT 3'UTR.

Example 7- Variation in the 3'UTR of MAPT Impacts Post-transcriptional Expression of Tau at Baseline and In Response to A β

Materials and Methods

RNA extraction was performed by disruption of fresh-frozen brain tissue by pulverization under liquid nitrogen, lysed in QIAzol and homogenized using a QIAshredder spin column (Qiagen, Valencia, CA). RNA was extracted using an RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using a First Strand cDNA Synthesis Kit (Origene, Rockville, MD), and used a template (1:4 dilution) in 20 μï reactions.

Primers and probes specific for 3R tau, 4R tau, total tau and GAPDH and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster, CA) were used for quantitative real-time polymerase chain reactions on a Mastercycler ep realplex (Eppendorf, Hauppauge, NY), using the following settings: 95°C for 10 minutes followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 minute. For QPCR of APP mRNA, FastStart Universal SYBR Green Master (Roche Applied Science) was used with primers described by Gilberto *et al.* (2008). The mRNA level was normalized to GAPDH.

The dual-luciferase 3'UTR reporter assay was performed using sequences covering the full-length MAPT 3'UTR that were PCR amplified from genomic DNA using the following primers:

Forward primer

5'-AATTCTAGGCGATCGCTGAGAAGCAGGGTTTGTGATCAGG-3 '; (SEQ ID NO: 4)

Reverse primer

5'-ATTTTATTGCGGCCAGCGGCCGCGGTGCGTGGGAAAGAACTTA-3 '; (SEQ ID NO: 5)

and cloned into the Xhol-Notl sites of the dual luciferase reported vector psiCHECK-2 (Promega, Madison, WI) using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA) downstream of Renilla luciferase. A full-length 3'UTR sequences of HI amplified from human brain genomic DNA from one TPD patient harboring the rs5820605 deletion, designated $Hl^{3'UTR\text{-}r_{s5820605}}$, one control with the insertion, designated $H1^{3'UTR}$, as well as one H2, designated $H2^{3'UTR}$ were inserted into the dual luciferase reporter system as shown in Figure 7. Luciferase reporter constructs were transfected into SH-SY5Y cells using NeuroPORTER (Genlantis, San Diego, CA). Luciferase activity was assayed using a Dual_-lo Luciferase Assay Kit (Promega) and expressed as the ratio of Renilla to firefly luciferase.

SH-SY5Y cells were grown either in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 medium (Cellgro) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% C02 and 95% air at 37°C.

Results

The potential that there may be risk-associated variants within the 3'UTR of MAPT in TPD prompted the consideration of alterations that occur in TPD at the mRNA level. Many cis-acting elements are contained within 3'UTRs that impact virtually all levels of gene expression.

QPCR analysis did not reveal any differences in the levels or ratios of 3R and 4R tau among TPD, AD and control (Figure 8a).

Using the reporter system, luciferase activity was compared between the three constructs described above (Figure 8b). Following normalization, the 3'UTR constructs displayed significantly different expression levels (p= 0.0004, one-way ANOVA). The $H2^{3'UTR}$ displayed the highest signal at baseline, while surprisingly the $H1^{3'UTR\_rs5820605}$ construct has the lowest luminosity, and the control $HI^{3'UTR}$, was intermediate. These findings are consistent with the hypothesis that genetic variation in the MAPT 3'UTR impacts post-transcriptional regulation of tau expression.

As shown in Example 2, there has been no significant amyloid accumulation in the brains of TPD patients. However, the possibility of a transient elevation of Aβ may trigger a cascade that ultimately leads to neurofibrillary degeneration and cell death. Using reporter gene constructs, the response of the different MAPT 3'UTRs to Aβ was determined.

A dramatic decrease in luciferase expression of cells transfected with the $H2^{3'UTR}$ construct (p=0.018, one -way ANOVA), and an increase in expression of both the $HI^{3'UTR-rs5820605}$ construct and the control $HI^{3'UTR}$, was seen in response to the exogenous application of 300 nM of Aβ (Figure 8c). There is no significant difference between the HI constructs, suggesting that the rs5820605 polymorphism does not mediate post-transcriptional alterations in tau expression in response to Aβ,

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CLAIMS

1. A method of screening, diagnosing, predicting, or identifying tangle-predominant dementia in a subject, comprising:
   a. obtaining biological tissue or bodily fluid from the subject;
   b. isolating a sample of protein from the biological tissue or bodily fluid;
   c. measuring the quantity of A β peptide or protein in the sample of protein; and
   d. comparing the quantity of A β peptide or protein in (c) with a reference value of the quantity of A β peptide or protein, the reference value representing a known diagnosis or prediction of Alzheimer's disease or normal cognitive function, and finding a deviation in the quantity of the A β peptide or protein measured in (c) from the reference value;

   wherein if the deviation in quantity of A β peptide or protein measured in (c) is decreased from or, lower or less than the reference value of the quantity of A β peptide or protein, then the subject can be determined, diagnosed, predicted or identified as having tangle-predominant dementia.

2. The method of claim 1, wherein the subject is human.
3. The method of claim 1, wherein the subject is suffering with cognitive impairment ranging from mild to severe or with pre-dementia in the prodromal phase.
4. The method of claim 1, wherein the biological tissue is brain, epidermal, blood or plasma.
5. The method of claim 1, wherein the bodily fluid is cerebrospinal fluid, saliva, plasma, sweat or urine.
6. The method of claim 1, wherein the quantity of A β peptide or protein in the sample of protein is measured using an antibody that recognizes or binds to A β peptide or protein.
7. The method of claim 1, wherein the level of A β peptide or protein in the sample of protein is measured by an assay selected from the group consisting of quantitative Western blots, immunoblots, quantitative mass spectrometry, enzyme-linked immunosorbent assays, radioimmunoassays, immunoradiometric assays, immunoenzymatic assays and sandwich assays.

8. The method of claim 1, wherein the A β peptide or protein being measured is A β42.

9. The method of claim 1, wherein the A β peptide or protein being meaured is A β40.

10. A kit for performing the method of claim 1, comprising (i) an antibody that recognizes or bind to the A β peptide or protein, for measuring the quantity of the A β peptide or protein in a sample from a subject, and (ii) a reference value of the A β peptide or protein or a means for establishing a reference value, wherein the reference value represents a known diagnosis or prediction for tangle-predominant dementia.

11. A method of screening, diagnosing, predicting, or identifying tangle-predominant dementia in a subject, comprising:

a. obtaining biological tissue or bodily fluid from the subject;

b. isolating a sample of protein from the biological tissue or bodily fluid;

c. measuring the quantity of N-terminal fragments of amyloid precursor protein in the sample of protein; and

d. comparing the quantity of N-terminal fragments of amyloid precursor protein in (c) with a reference value of the quantity of N-terminal fragments of amyloid precursor protein, the reference value representing a known diagnosis or prediction normal cognitive function, and finding a deviation in the quantity of the N-terminal fragments of amyloid precursor protein measured in (c) from the reference value;

wherein a deviation in the quantity of the N-terminal fragments of amyloid precursor protein measured in (c) from the reference value of the quantity of the N-terminal fragments of amyloid precursor, determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

12. The method of claim 11, wherein the subject is human.

13. The method of claim 11, wherein the subject is suffering with cognitive impairment ranging from mild to severe or with pre-dementia in the prodromal phase.

14. The method of claim 11, wherein the biological tissue is brain, epidermal, blood or plasma.

15. The method of claim 11, wherein the bodily fluid is cerebrospinal fluid, saliva, plasma, sweat or urine.

16. The method of claim 11, wherein the protein is isolated and purified from the biological tissue or bodily fluid.
17. The method of claim 11, wherein the quantity of N-terminal fragments of amyloid precursor protein in the sample of protein is measured using an antibody that recognizes or binds to the N-terminal fragments of amyloid precursor protein.
18. The method of claim 11, wherein the level of N-terminal fragments of amyloid precursor protein in the sample of protein is measured by an assay selected from the group consisting of quantitative Western blots, immunoblots, quantitative mass spectrometry, enzyme-linked immunosorbent assays, radioimmunoassays, immunoradiometric assays, immunoenzymatic assays and sandwich assays.
19. The method of claim 11, wherein the N-terminal fiagment of amyloid precursor protein being measured is sAPPa, and wherein if the quantity of sAPPa in the sample of protein from the subject is increased from, or higher or greater than the reference value of sAPPa, then the subject can be determined, diagnosed, predicted or identified as having tangle-predominant dementia.
20. The method of claim 11, wherein the N-terminal fragment of amyloid precursor protein being measured is sAPPp, and wherein if the quantity of sAPPP in the sample of protein from the subject is decreased from, or lower or less than the reference value of sAPPp, then the subject can be determined, diagnosed, predicted or identified as having tangle-predominant dementia.
21. A kit for performing the method of claim 11, comprising (i) an antibody or antibodies that recognizes or binds to the sAPPa, and sAPPp for measuring the quantity of the sAPPa, and sAPPp in a sample from a subject, and (ii) a reference value of the sAPPa, and sAPPp or a means for establishing a reference value, wherein the reference value represents a known diagnosis or prediction for tangle-predominant dementia.
22. A method of screening, diagnosing, predicting or identifying tangle-predominant dementia in a subject, comprising:
    a. obtaining biological tissue or bodily fluid from the subject;
    b. isolating and purifying a sample of nucleic acid from the biological tissue or bodily fluid; and

c. detecting the presence of Apoliprotein E alleles, ε2, ε3, or ε4 in the sample of nucleic acid by sequencing the nucleic acid sample obtained from the biological tissue or bodily fluid of the subject, and comparing the sequence of the nucleic acid sample to the known reference nucleic acid sequences of Apoliprotein alleles ε2, ε3, or ε4,

wherein the presence of Apoliprotein E alleles ε2 or ε3 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia, or the absence of the Apoliprotein E allele ε4 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

23. The method of claim 22, wherein the subject is human.

24. The method of claim 22, wherein the subject is suffering with cognitive impairment ranging from mild to severe or with pre-dementia in the prodromal phase.

25. The method of claim 22, wherein the biological tissue is brain, epidermal, blood or plasma.

26. The method of claim 22, wherein the bodily fluid is cerebrospinal fluid, saliva, plasma, sweat or urine.

27. The method of claim 22, wherein the nucleic acid is RNA, cDNA or genomic DNA.

28. The method of claim 22, wherein the presence of the Apolipoprotein alleles is detected by amplifying the Apolipoprotein E gene in the sample of nucleic acid from the biological tissue or bodily fluid of the subject with a primer.

29. A method of screening, diagnosing, predicting or identifying tangle-predominant dementia in a subject, comprising:

a. obtaining biological tissue or bodily fluid from the subject;

b. isolating and purifying a sample of nucleic acid from the biological tissue or bodily fluid; and

c. detecting the presence of Apoliprotein E alleles, ε2, ε3, or ε4 in the sample of nucleic acid;

wherein the presence of the Apoliprotein E alleles, ε2, ε3, or ε4 in the sample of nucleic acid is detected by an assay selected from the group consisting of (a) hybridizing a Apoliprotein E allele, ε2, ε3, or ε4 gene probe to the nucleic acid sample, and detecting the presence of hybridization products, (b) hybridizing an allele-specific probe to nucleic acid sample and detecting the

presence of hybridization products in the sample, (c) amplifying all or part of the Apo E allele from the nucleic acid sample to produce an amplified sequence and sequencing the amplified sequence, (d) amplifying all or part of the Apo E allele from the nucleic acid sample using primers for a specific Apo E alleles, ε2, ε3, or ε4 and determining the presence of a hybridization product in the sample, (e) molecularly cloning all or part of the Apo E allele from the nucleic acid sample to produce a cloned sequence and sequencing the cloned sequence, (f) amplification of Apo E allele sequences in the nucleic acid sample and hybridization of the amplified sequences to nucleic acid probes which comprise the specific Apo E allele sequences, and (g) in situ hybridization of the Apo *E* allele of the nucleic acid sample with nucleic acid probes which comprise the Apo E alleles; and wherein the presence of Apoliprotein E alleles ε2 or ε3 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia, or the absence of the Apoliprotein E allele ε4 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

30. The methods of claim 21 or 28, wherein the presence of all three Apoliprotein E alleles, ε2, ε3, and ε4 are detected, and wherein the presence of the ε2 or ε3 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia, and the absence of the Apoliprotein E alleles ε4 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

31. A method of screening, diagnosing, predicting or identifying tangle-predominant dementia in a subject, comprising:

a. obtaining biological tissue or bodily fluid from the subject;

b. isolating and purifying a sample of nucleic acid from the biological tissue or bodily fluid; and

c. detecting the presence of H1 haplotype of the *MAPT* locus in the sample of nucleic acid by sequencing the nucleic acid sample obtained from the biological tissue or bodily fluid of the subject, and comparing the sequence of the nucleic acid sample to the known reference nucleic acid sequences of the H1 haplotype of the *MAPT* locus,

wherein the presence of H I haplotype of the *MAPT* locus determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

32. The method of claim 31, wherein the subject is human.
33. The method of claim 31, wherein the subject is suffering with cognitive impairment ranging from mild to severe or with pre-dementia in the prodromal phase.
34. The method of claim 31, wherein the biological tissue is brain, epidermal, blood or plasma.
35. The method of claim 31, wherein the bodily fluid is cerebrospinal fluid, saliva, plasma, sweat or urine.
36. The method of claim 31, wherein the nucleic acid is RNA, cDNA or genomic DNA.
37. The method of claim 31, wherein the presence of the H I haplotype of the *MAPT* locus is detected by amplifying the H I haplotype of the *MAPT* locus in the sample of nucleic acid from the biological tissue or bodily fluid of the subject with a primer.
38. The method of claim 31, wherein the sequence of the nucleic acid sample of the subject is compared to the reference nucleic acid sequence of SEQ ID NO: 6.
39. A method of screening, diagnosing, predicting or identifying tangle-predominant dementia in a subject, comprising:
    a. obtaining biological tissue or bodily fluid from the subject;
    b. isolating and purifying a sample of nucleic acid from the biological tissue or bodily fluid; and
    c. detecting the presence of H I haplotype of the *MAPT* locus in the sample of nucleic acid;

    wherein the presence of the H I haplotype of the *MAPT* locus in the sample of nucleic acid is detected by an assay selected from the group consisting of (a) hybridizing a H I haplotype probe to the nucleic acid sample, and detecting the presence of hybridization products, (b) hybridizing an allele-specific probe to nucleic acid sample and detecting the presence of hybridization products in the sample, (c) amplifying all or part of the *MAPT* locus from the nucleic acid sample to produce an amplified sequence and sequencing the amplified sequence, (d) amplifying all or part of the *MAPT* locus from the nucleic acid

sample using primers for the H I haplotype of the *MAPT* locus and determining the presence of a hybridization product in the sample, (e) molecularly cloning all or part of the *MAPT* locus from the nucleic acid sample to produce a cloned sequence and sequencing the cloned sequence, (f) amplification of *MAPT* locus sequences in the nucleic acid sample and hybridization of the amplified sequences to nucleic acid probes which comprise the H I haplotype of the *MAPT* locus and (g) in situ hybridization of the *MAPT* locus of the nucleic acid sample with nucleic acid probes which comprise the H I haplotype of the *MAPT* locus; and

wherein the presence of the H I haplotype of the *MAPT locus* determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

40. A method of screening, diagnosing, predicting or identifying tangle-predominant dementia in a subject, comprising:
    a. obtaining biological tissue or bodily fluid from the subject;
    b. isolating and purifying a sample of nucleic acid from the biological tissue or bodily fluid; and
    c. detecting the presence of the polymorphism designated rs35 134565 with the nucleotide sequence comprising SEQ ID NO: 1 and the polymorphism designated rs5 820605 with the nucleotide sequence comprising SEQ ID NO: 2, in the sample of nucleic acid by sequencing the nucleic acid sample obtained from the biological tissue or bodily fluid of the subject, and comparing the sequence of the nucleic acid sample to the known reference nucleic acid sequences of the polymorphisms designated rs35134565 and rs5820605,

    wherein the presence of the polymorphism designated rs3 5144565 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia, or the absence of the polymorphism designated rs5 820605 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

41. The method of claim 40, wherein the subject is human.

42. The method of claim 40, wherein the subject is suffering with cognitive impairment ranging from mild to severe or with pre-dementia in the prodromal phase.

43. The method of claim 40, wherein the biological tissue is brain, epidermal, blood or plasma.
44. The method of claim 40, wherein the bodily fluid is cerebrospinal fluid, saliva, plasma, sweat or urine.
45. The method of claim 40, wherein the nucleic acid is RNA, cDNA or genomic DNA.
46. The method of claim 40, wherein the presence of the polymorphisms is detected by amplifying the 3'UTR of the *MAPT* locus in the sample of nucleic acid from the biological tissue or bodily fluid of the subject with a primer.
47. A method of screening, diagnosing, predicting or identifying tangle-predominant dementia in a subject, comprising:
    a. obtaining biological tissue or bodily fluid from the subject;
    b. isolating and purifying a sample of nucleic acid from the biological tissue or bodily fluid; and
    c. detecting the presence of the polymorphisms designated rs35134565 and rs5820605 in the sample of nucleic acid;

    wherein the presence of the polymorphisms designated rs35134565 and rs5820605in the sample of nucleic acid is detected by an assay selected from the group consisting of (a) hybridizing a probe to the nucleic acid sample, and detecting the presence of hybridization products, (b) hybridizing an allele-specific probe to nucleic acid sample and detecting the presence of hybridization products in the sample, (c) amplifying all or part of the 3'UTR of *MAPT* locus from the nucleic acid sample to produce an amplified sequence and sequencing the amplified sequence, (d) amplifying all or part of the 3'UTR from *MAPT* locus from the nucleic acid sample using primers for the polymorphisms designated rs35134565 and rs5820605 and determining the presence of a hybridization product in the sample, (e) molecularly cloning all or part of the 3' UTR of *MAPT* locus from the nucleic acid sample to produce a cloned sequence and sequencing the cloned sequence, (f) amplification of the 3'UTR of *MAPT* locus sequences in the nucleic acid sample and hybridization of the amplified sequences to nucleic acid probes which comprise the polymorphisms designated rs35134565 and rs5820605and (g) in situ hybridization of the 3' UTR of *MAPT* locus of the nucleic acid sample with nucleic acid probes

which comprise the polymorphisms designated rs35 134565 and rs5 820605; and

wherein the presence of the polymorphism designated rs3 5134565 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia, and the absence of the polymorphism designated rs5820605 determines, diagnoses, predicts or identifies the subject as having tangle-predominant dementia.

Fig 1

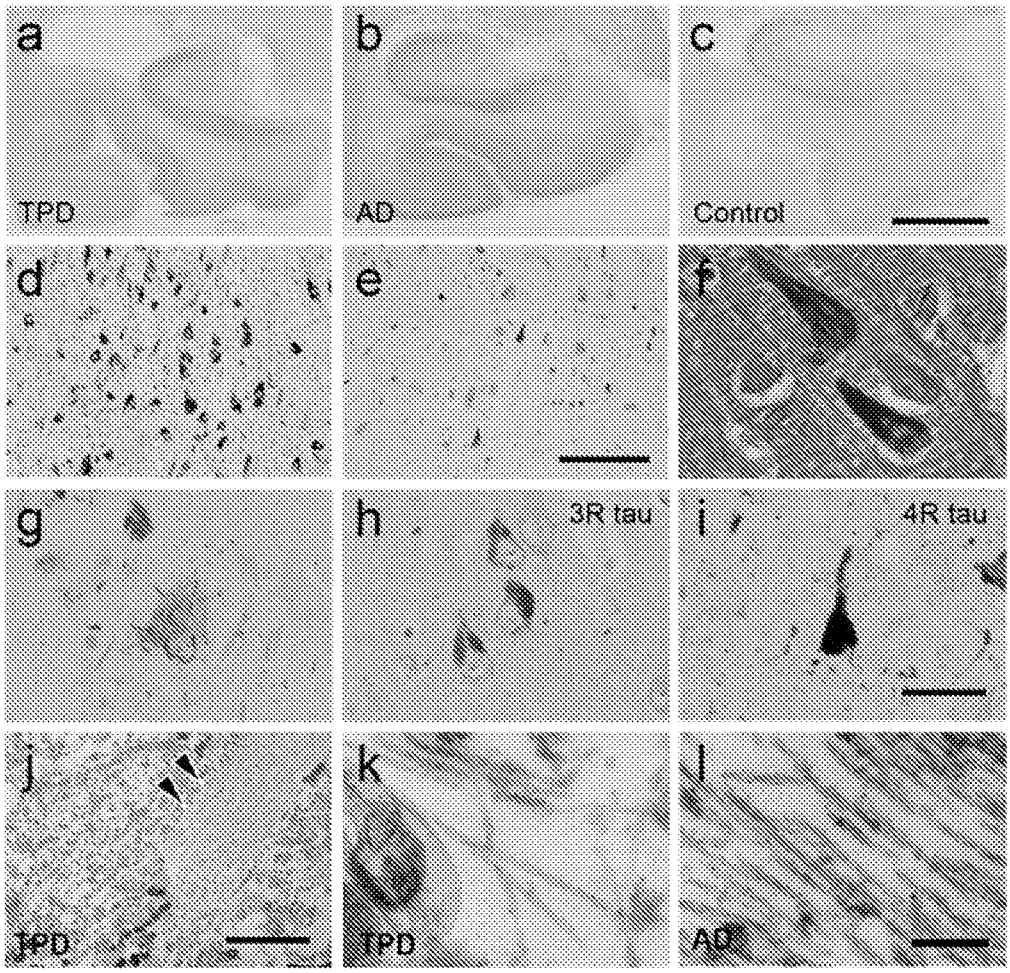


Fig 2

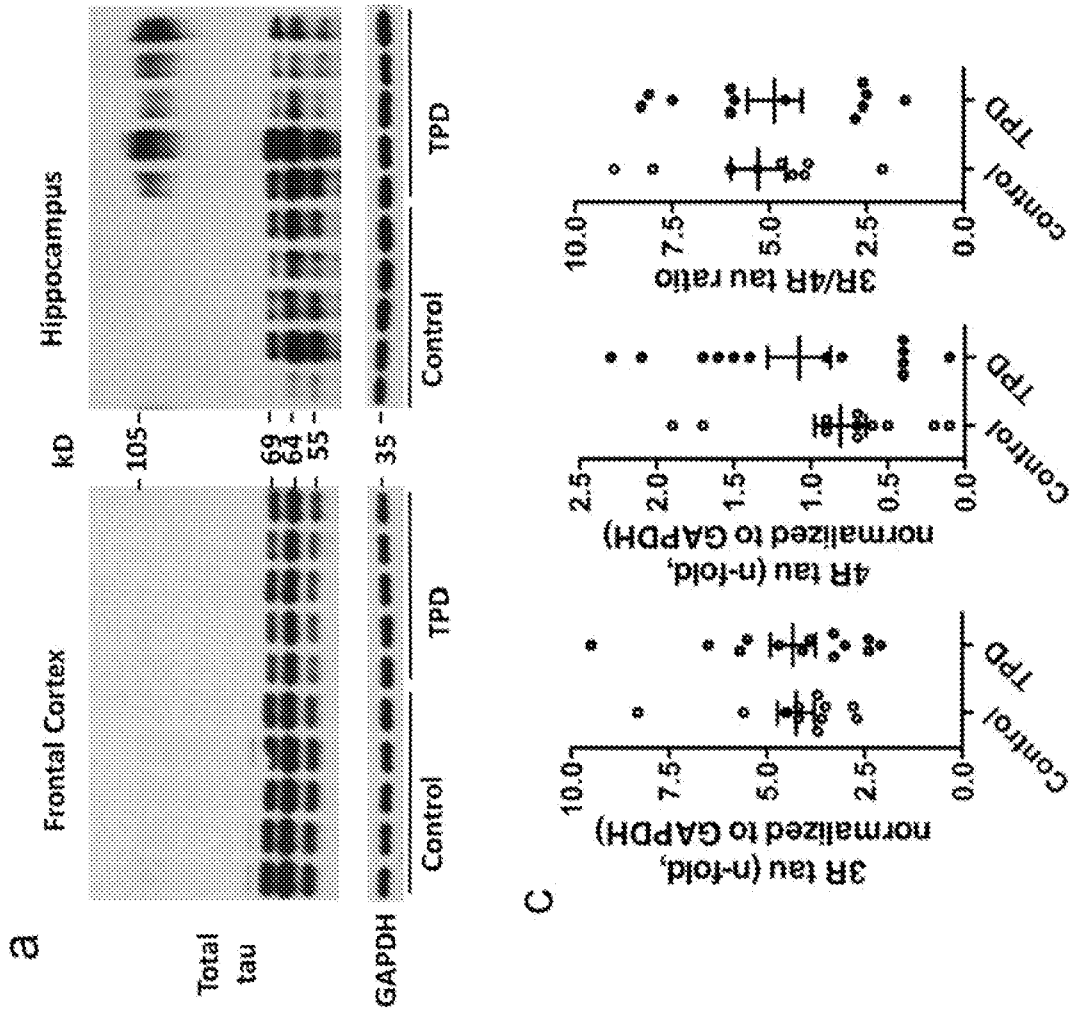

a
Frontal Cortex
Hippocampus
kD
105
69
64
55
35
Total tau
GAPDH
Control
TPD
b
Frontal Cortex
3R tau
GAPDH
4R tau
GAPDH
69
64
55
35
Control
TPD
c
3R tau (n-fold, normalized to GAPDH)
4R tau (n-fold, normalized to GAPDH)
3R/4R tau ratio
10.0
7.5
5.0
2.5
0.0
2.5
2.0
1.5
1.0
0.5
Control
TPD
d
Insoluble tau
kD
105
69
64
55
AD
Control
TPD
e
AD
TPD
periodicity = 65 – 70 nm
thickness = 9 – 18 nm

Fig 3

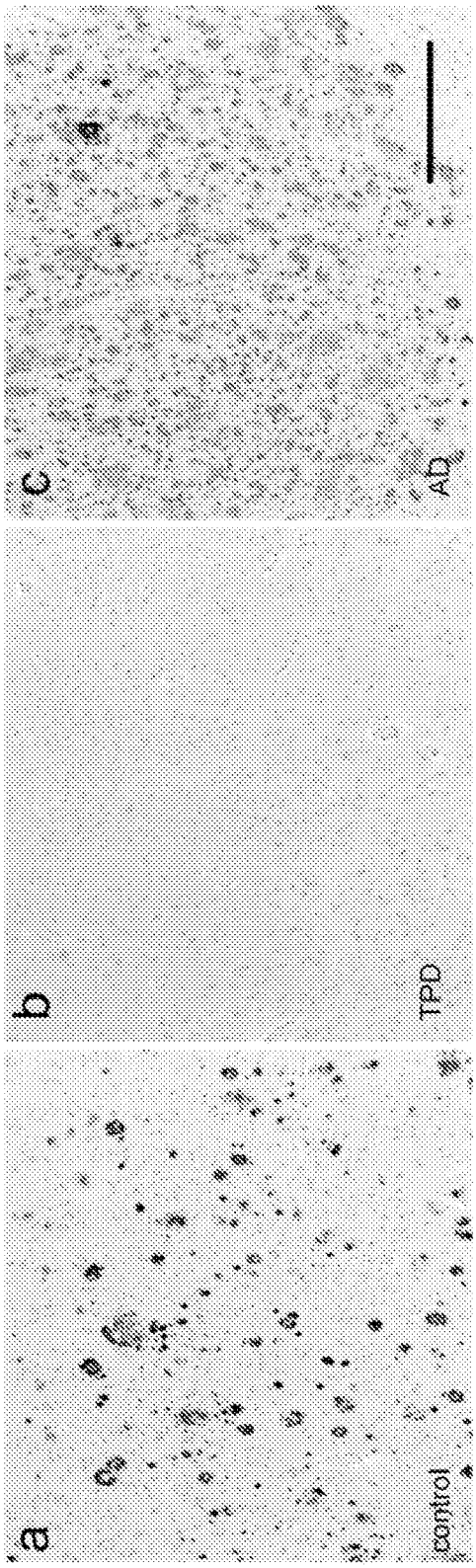


Fig 3

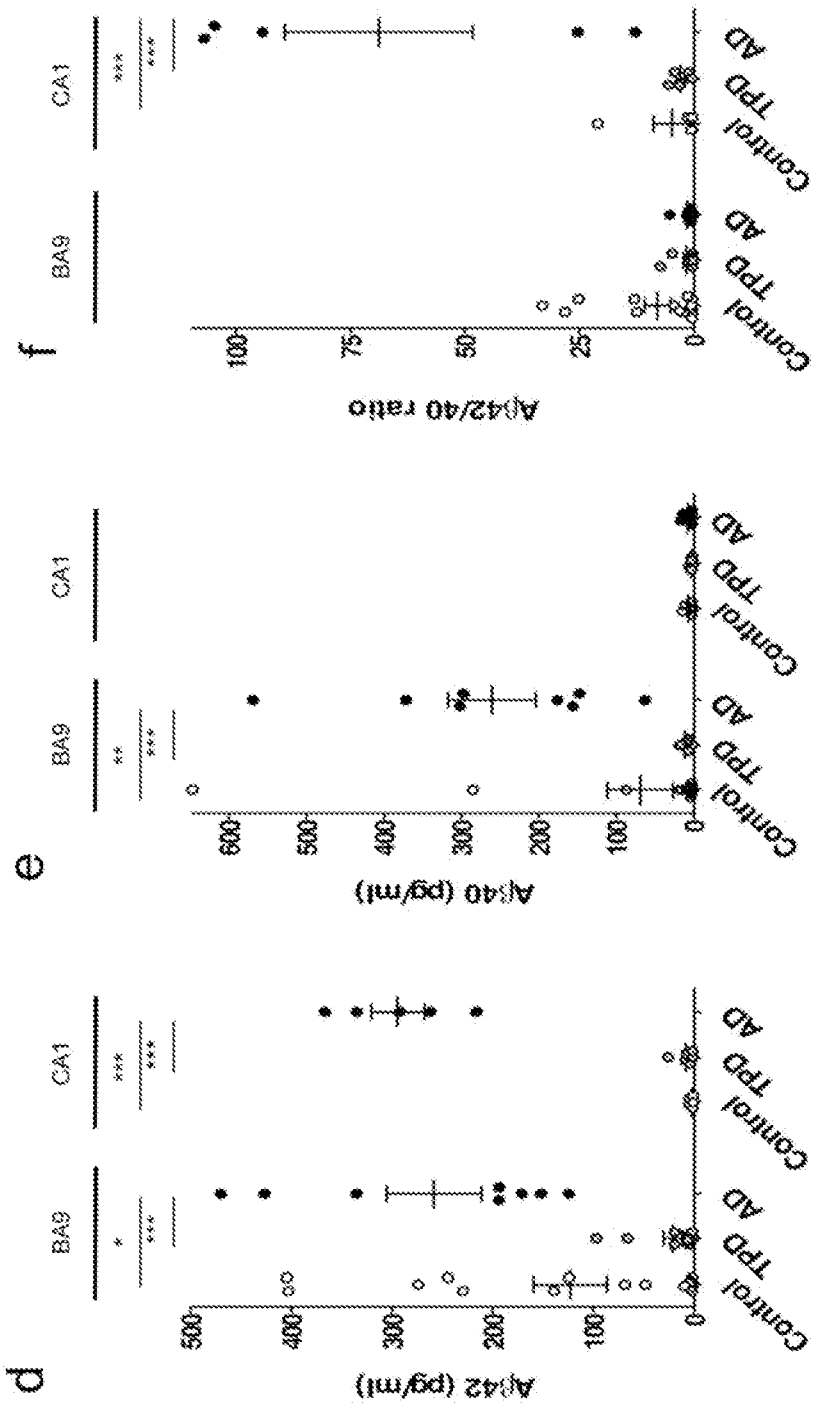
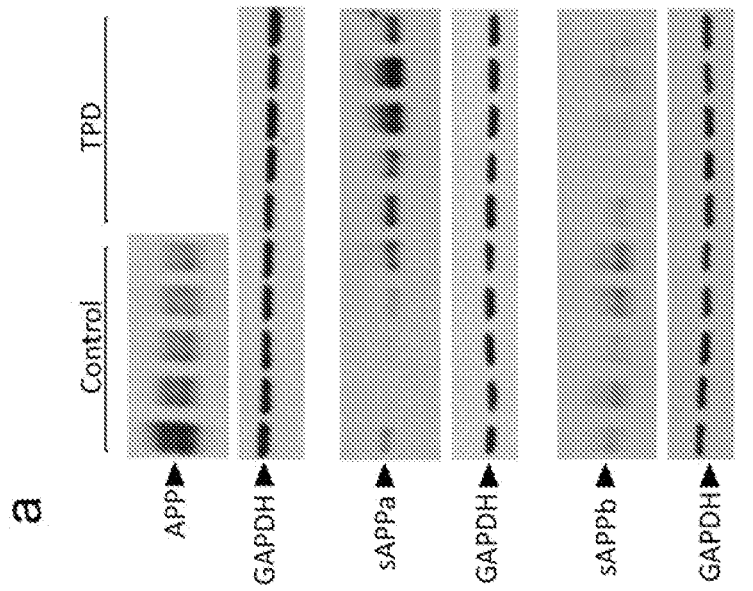
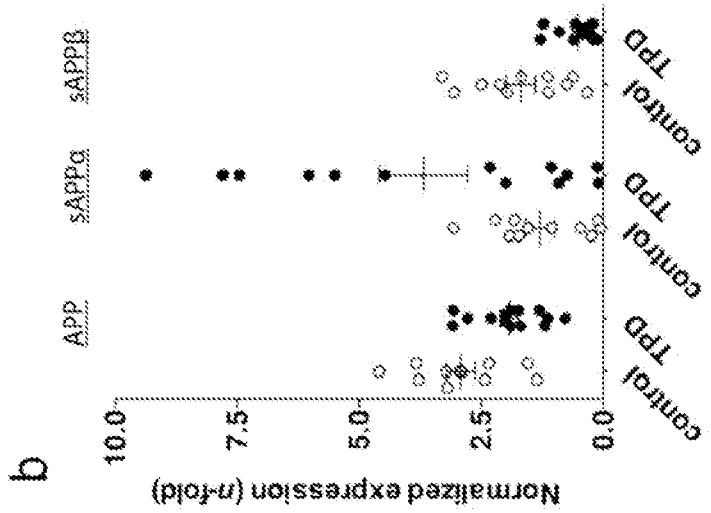


Fig 4


a
Control
TPD
APP
GAPDH
sAPPa
GAPDH
sAPPb
GAPDH


b
Normalized expression (n-fold)
10.0
7.5
5.0
2.5
0.0
APP
sAPPα
sAPPβ
control
TPD

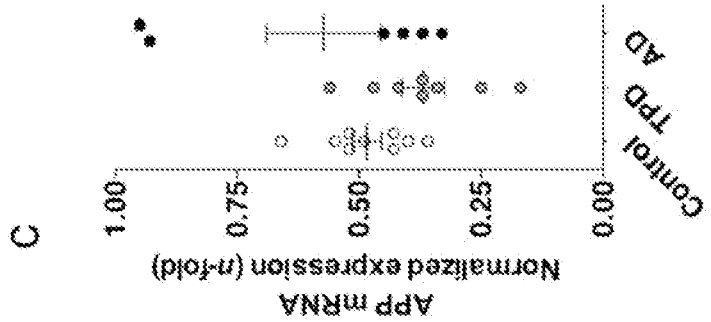
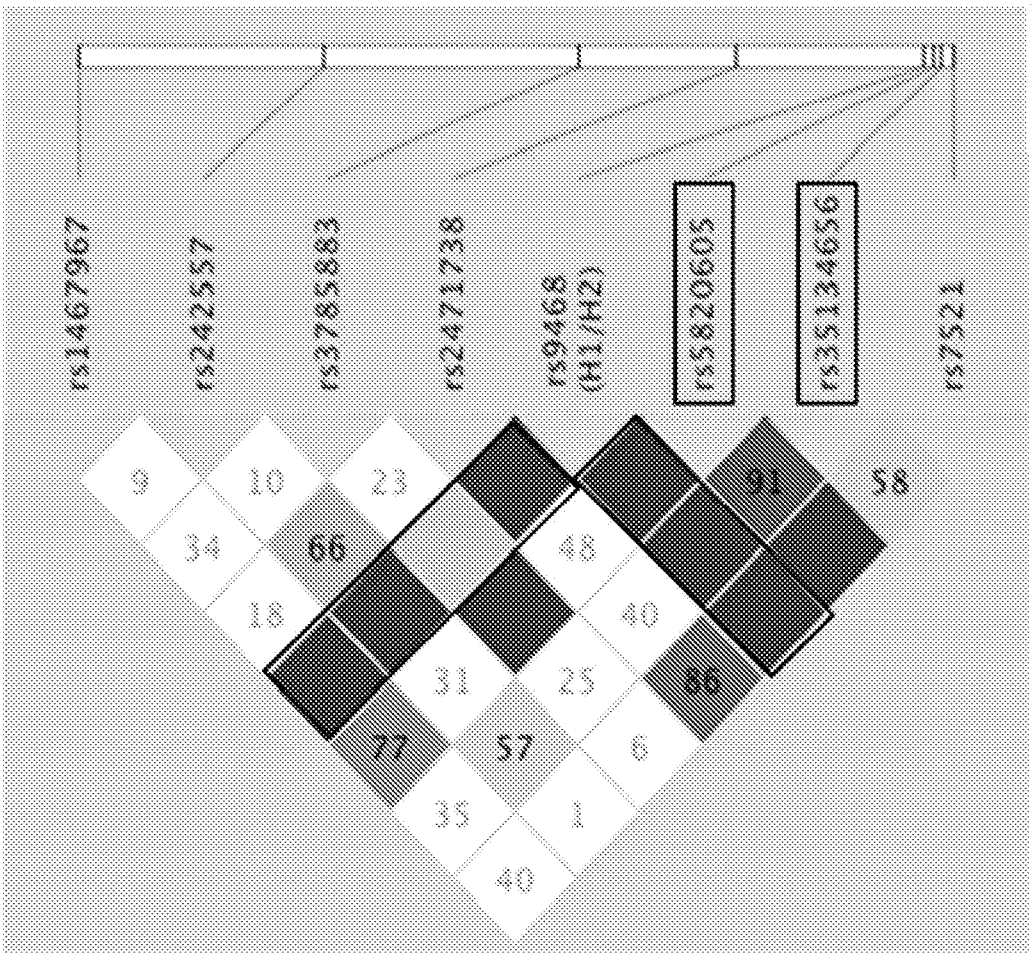

c
APP mRNA
Normalized expression (n-fold)
1.00
0.75
0.50
0.25
0.00
control
TPD
AD

Fig 5


rs14679067
rs2422557
rs27858883
rs24711738
rs9468 (H1/H2)
rs58206605
rs35134659
rs7521
9
10
23
34
66
18
48
40
31
25
27
57
6
35
1
40
94
58
66

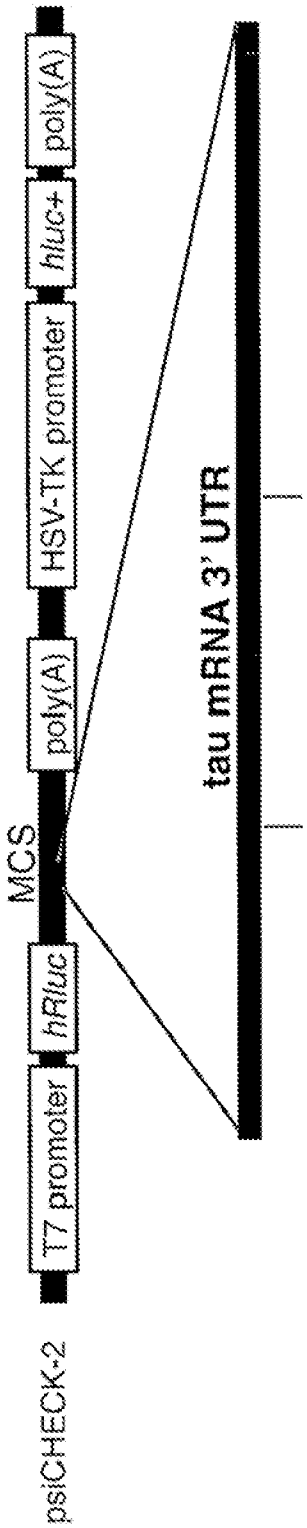

psiCHECK-2
T7 promoter
hRluc
MCS
poly(A)
HSV-TK promoter
hluc+
poly(A)
tau mRNA 3' UTR

Fig. 7

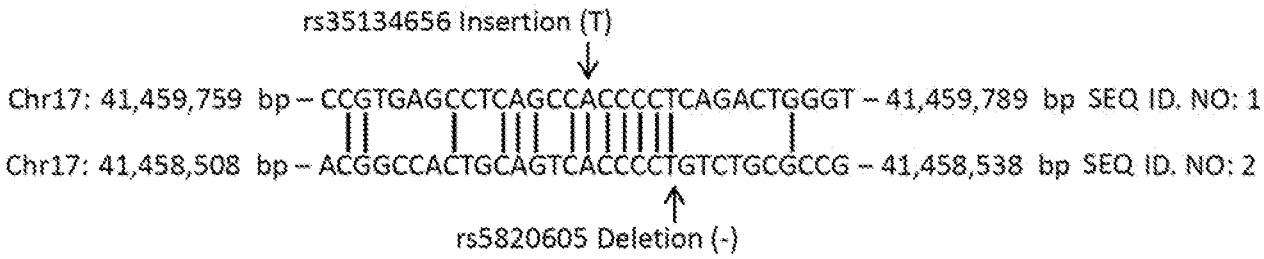

rs35134656 Insertion (T)
Chr17: 41,459,759 bp – CCGTGAGCCTCAGCCACCCCTCAGACTGGGT – 41,459,789 bp SEQ ID. NO: 1
Chr17: 41,458,508 bp – ACGGCCACTGCAGTCACCCCTGTCTGCGCCG – 41,458,538 bp SEQ ID. NO: 2
rs5820605 Deletion (-)

Fig 6

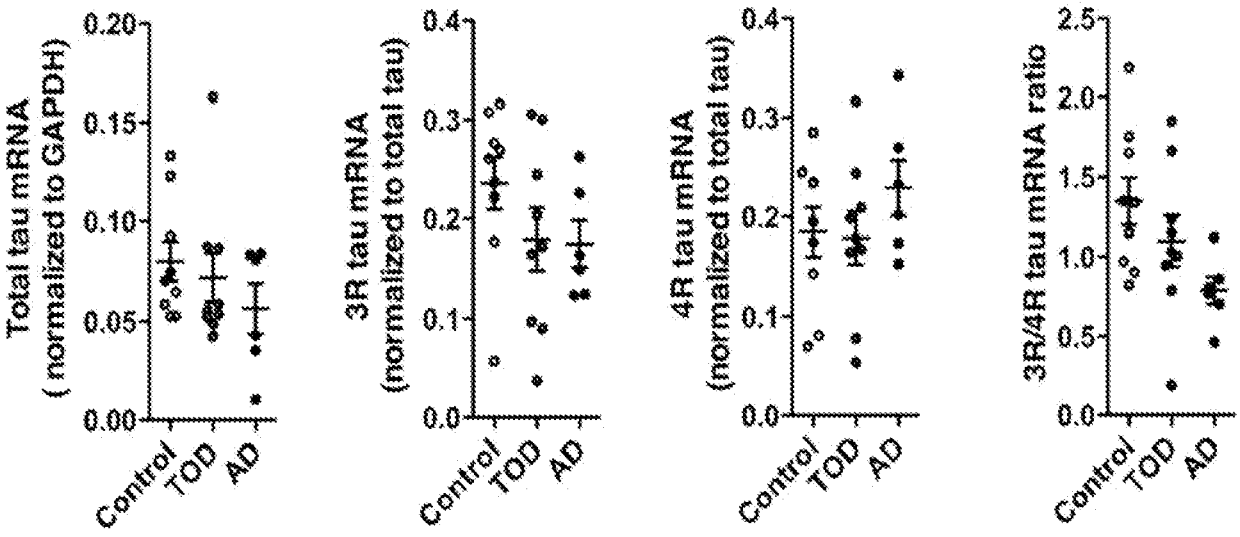

Total tau mRNA (normalized to GAPDH)
0.20
0.15
0.10
0.05
0.00
Control
TOD
AD
3R tau mRNA (normalized to total tau)
0.4
0.3
0.2
0.1
0.0
4R tau mRNA (normalized to total tau)
3R/4R tau mRNA ratio
2.5
2.0
1.5
1.0
0.5

Fig 8a

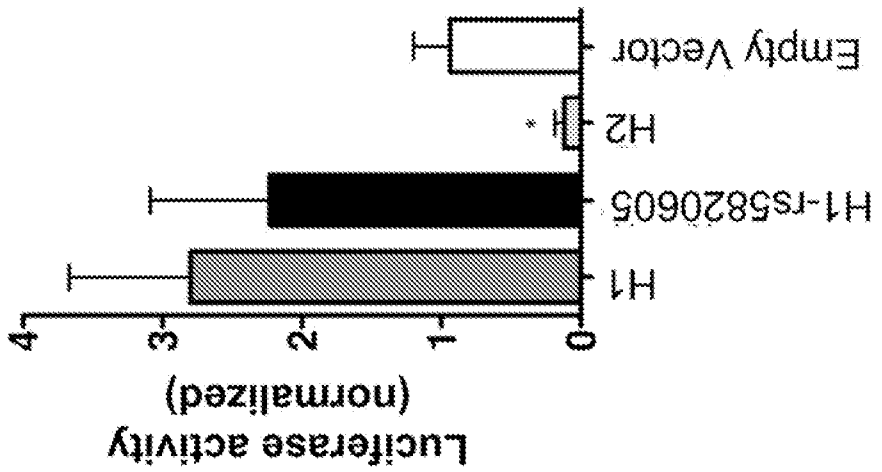

Luciferase activity
(normalized)
0
1
2
3
4
H1
H1-rs58206605
H2
*
Empty Vector

Fig 8c

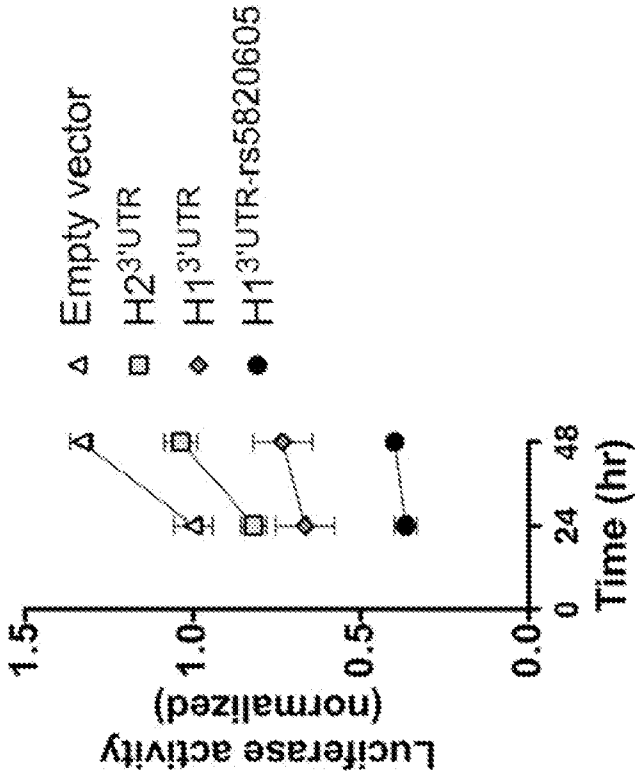

Luciferase activity
(normalized)
0.0
0.5
1.0
1.5
0
24
48
Time (hr)
Empty vector
H2 3'UTR
H1 3'UTR
H1 3'UTR-rs58206605

Fig 8b

专利名称(译)	以缠结为主的痴呆症的生物标志物		
公开(公告)号	EP2864782A4	公开(公告)日	2016-08-03
申请号	EP2013807522	申请日	2013-06-21
[标]申请(专利权)人(译)	纽约市哥伦比亚大学理事会		
申请(专利权)人(译)	哥伦比亚大学纽约市受托人		
当前申请(专利权)人(译)	哥伦比亚大学纽约市受托人		
[标]发明人	CRARY JOHN		
发明人	CRARY, JOHN		
IPC分类号	G01N33/53 C12Q1/68 G01N33/68		
CPC分类号	G01N33/6896 C12Q1/6883 C12Q2600/112 C12Q2600/156 C12Q2600/158 G01N33/6854 G01N2333/4709 G01N2800/2814 G01N2800/2821		
优先权	61/662644 2012-06-21 US		
其他公开文献	EP2864782A2		
外部链接	Espacenet		

摘要(译)

本发明涉及筛选，鉴定和诊断缠结型主要痴呆（TPD）的领域。具体地，本发明提供了用于该疾病的各种生物标志物，以及使用这些生物标志物通过将TPD患者与经典阿尔茨海默病（AD），以及前驱AD和轻度认知障碍区分开来对阿尔茨海默氏型痴呆患者进行正确分类的方法。由于AD。