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Single molecule profiling of tau gene expression in Alzheimer's disease

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Abstract

Tau is a microtubule-associated protein that is important for establishing and maintaining neuronal morphology. In addition to its role in normal cells, tau protein is involved in many neurodegenerative diseases, e.g. Alzheimer's disease (AD) and frontotemporal dementia, as the main component of intraneuronal aggregates. Alternative splicing of tau gene in the brain can give rise to at least six protein variants. A causative role of skewed tau exon 10 inclusion has been defined in frontotemporal dementia; however, no link was established between the aberrant splicing of tau and AD. Here, we applied a single-molecule-based technology, polymerase colony or *polony*, to simultaneously monitor tau splicing variant and haplotype profile in sporadic AD and normal brains. We found that the coordinated expression of tau exons 2 and 10 is altered in AD. Additional investigations of *cis* and *trans* mechanisms of this observation revealed a decreased protein expression of a known tau splicing factor, htra2-beta-1 in AD, thereby implicating a *trans* mechanism. Our results demonstrate that dysregulation of combinatorial splicing might serve as a signature for aging-related diseases, and the *polony* assay could be widely adapted for the study of other tauopathies. Furthermore, splicing-based therapeutics is an emerging area of drug development, and a well-defined and quantitative assay for monitoring single-gene transcriptome will be relevant for such development.

Keywords: Alzheimer's disease, splicing, tau.
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Alternative pre-mRNA splicing is a major contributor of mammalian proteome diversity. Combinatorial usage of multiple alternatively spliced exons of a transcript can lead to exponential accumulation of protein variants, which may play distinct roles in establishing and maintaining cellular identity (Lander *et al.* 2001). One such locus encodes for the microtubule-associated protein, tau. In the human CNS, at least six tau isoforms can be produced as the result of combinatorial inclusion of its exons 2, 3, and 10 (Fig. 1a). Although the function of individual tau variants remains to be fully determined, dysregulation of tau splicing is often observed in neurodegenerative diseases with aberrant tau deposition, including frontotemporal dementia (FTD), Pick's disease, and progressive supranuclear palsy (Brandt *et al.* 2005). In FTD, several mutations in the tau gene have been shown to not only promote tau aggregation but also skew tau splicing (D'Souza and Schellenberg 2005), prompting the investigation of tau splicing in other tau pathology-related disorders like Alzheimer's disease (AD).

Alternative splicing of the tau gene in AD patients had been previously examined using *in situ* hybridization (Goedert *et al.* 1989a,b), microarray analysis (Ginsberg *et al.* 2006), and PCR-based approaches (Chambers *et al.* 1999; Yasojima *et al.* 1999; Boutajangout *et al.* 2004; Umeda *et al.* 2004; Connell *et al.* 2005; Glatz *et al.* 2006; Ingelsson *et al.* 2006); most of these studies did not detect differences in AD, although several experiments suggested an increase in exon 10 in tangle affected neurons or brain areas (Yasojima *et al.* 1999; Glatz *et al.* 2006; Ginsberg *et al.* 2006). The different

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Abbrevations used: AD, Alzheimer's disease; bp, base pair; dNTP, deoxynucleotide; htra2β1, htra2-beta-1; FTD, frontotemporal dementia; GFAP, glial fibrillary acidic protein; LCM, laser capture microscopy; polony, polymerase colony; QPCR, quantitative PCR; SNP, single nucleotide polymorphism.

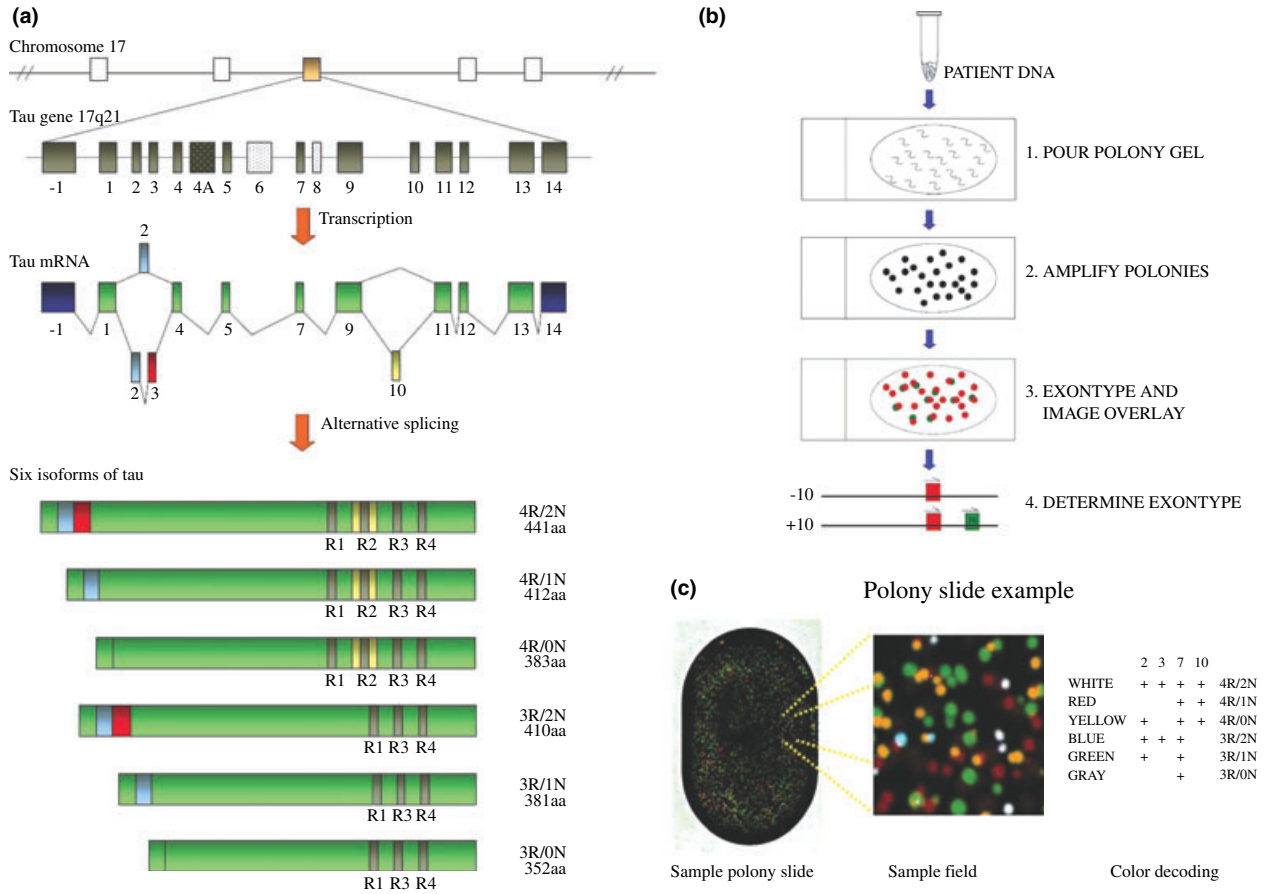

Fig. 1 Schematic diagram of the tau locus and *polony* technique. (a) The tau gene is located on human chromosome 17q21. Alternative splicing of exons 2, 3, and 10 of the transcript in the CNS creates six isoforms. (b) *Polony* exon profiling. The general steps of *polony* technique are shown. The template is a cDNA sample which is cast in acrylamide gel with immobilized forward primers to a customized microscope slide. Next, the gene of interest (tau) is amplified with the addition of PCR reagents (+ reverse primer) and followed by denaturation yielding single stranded polonies. These polonies can be later exon typed by hybridizing exon-specific fluorescent probes as shown and scanned on a microarray reader. (c) Representative *polony* image. The oval area shows a low magnification image of a *polony* slide adjacent to higher magnification image of a selected field. This sample field shows six different colored spots (each one representing a *polony*) and a color decoding legend is shown allowing the identification of specific tau isoforms.

results may be due to the complexity of the tau transcriptome and the limited resolution of the conventional assays to unambiguously monitor its variant profile. First, the sizes of alternatively spliced tau exons are nearly identical (exon 2: 87 bp, exon 3: 87 bp, and exon 10: 93 bp), hence, tau splicing variants are unlikely to be resolved by their mobility in gel electrophoresis. To circumvent the problem, many studies attempted to monitor the splicing of one exon at a time. However, the combinatorial information on individual tau transcripts is lost by such manipulation. Second, intensity-based (analog) quantitation is more susceptible to experimental noise and thereby less viable for detecting subtle changes. Third, noise intrinsic to PCR such as cross-hybridization of amplification primers because of the repetitive nature of the sequence in/around the microtubule-binding domains could impact their results (Takanashi *et al.* 2002). Although several improved methodologies have been developed to monitor tau alternative splicing, they overcome some, but not all, of the technical challenges (Sergeant *et al.* 2001; Connell *et al.* 2005; Ginsberg *et al.* 2006). One solution for the study of tau splicing or combinatorial splicing in general is to determine the exon composition of each individual transcript of interest, which demands a single-molecule-based technology.

We have developed a polymerase colony (*polony*)-based exon profiling platform that allows for quantitatively monitoring combinatorial splicing at the single-molecule level (Zhu *et al.* 2003). The assay is performed in three steps: creation of an acrylamide gel matrix with randomly arrayed DNA templates, in-gel PCR amplification, and finally an exon-specific oligonucleotide hybridization/single base extension (Fig. 1b). Because individual molecules are amplified in

parallel, interference between different molecules can be largely eliminated (Aach and Church 2004). Each *polony* has a fixed 'address' on a given slide, therefore multiple features of a given molecule [e.g. exon and single nucleotide polymorphism (SNP)] can be sequentially examined, and the exon composition of each molecule can be precisely determined. In contrast to analog approaches, quantitation with the *polony* platform is digital and determined by the number of molecules that share the same sequence color signature (Fig. 1c).

The aim of our study was to examine the role of tau splicing in the context of tau genetics in AD through the use of the *polony* technology to simultaneously profile tau isoforms and the tau haplotype in sporadic AD and normal brains. In this study, we found that the splicing of tau exons 2 and 10 is altered in AD, thereby placing it on the growing list of splicing-related disorders. Our additional investigations of *cis* and *trans* mechanisms for the aberrant tau splicing revealed a decrease in protein expression of a splicing factor, htra2-beta-1 (htra2β1), in AD, implicating this splicing factor in neurodegenerative disease.

Methods

Oligonucleotides

All primers used to perform *polony* amplification and exon profiling were previously described (Zhu *et al.* 2003). The names and sequences of the oligonucleotides are as follows:

Primer name	Primer sequence	tau exon
tau_Fac Acrydite linked	GGCTACACCATGCACCAAGACCA	Exon 1
tau_Rev	GTTGCCTAATGAGCCACACTTGGAG	Exon 11
tau_E2R	Cy3/TTCAGAGCCCGGTTCCTCAGATC	Exon 2
tau_E3R	Cy5/CCTCATCCACTAAGGGTGCTGTCAC	Exon 3
tau_E7R	Cy3/GGTGTGGCGATCTTCGTTTTACCA	Exon 7
tau_E10R	Cy5/GCCTCCCGGGACGTGTTTGATATT	Exon 10
Exon 9 N255N seq	AGTGGAGCCGATCTTGGACTTGAC	Exon 9

The sequencing primer for the silent SNP was at codon position N255N (AA**T**-H1/AA**C**-H2) of the tau 441 isoform [Exon 9 N255N seq] for haplotype (Baker *et al.* 1999) and allele bias analyses.

Polony amplification

A total of 46 subjects were used for the tau pre-mRNA expression study. Temporal neocortical brain tissue from AD (80.7 ± 6.7 years, $n = 22$,) and normal subjects (78.6 ± 10.7 years, $n = 20$) were used and have been previously described (Ingelsson *et al.* 2004; Bertram *et al.* 2005). Neocortical tissue from FTD ($n = 2$) (both cases had the tau exon 10+3 mutation described previously, Bernardino Ghetti unpublished data) (Spillantini *et al.* 1998) was used as a positive control. The total RNA of the subjects was extracted from frozen brain tissue using Trizol (Invitrogen, Carlsbad, CA, USA) followed by DNase treatment (Ambion, Austin, TX, USA). Reverse transcription was carried out with Superscript II–RNase H- (Invitrogen) using random hexamers. RNAs were then removed by NaOH digestion, and cDNA was further purified through QIAquick column (Qiagen, Valencia, CA, USA). The genomic DNA of the subjects was extracted from frozen brain tissue of AD and control subjects by using a genomic DNA extraction kit according to manufacturer protocols (Qiagen).

The *polony* amplification was performed as previous described (Zhu *et al.* 2003), Briefly, the three-step process involves the creation of the gel matrix on a slide, the infusion of PCR reagents, and a PCR step in a Slide Cycling '16/16' Dual PCR Block (MJ Research). The detailed protocol is also available at the Church lab *polony* website, http://arep.med.harvard.edu/Polonator/.

The gel mix contained (Tris–HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 7.5% acrylamide, 0.35% DATD, 0.035% Bis-acrylamide, 1 μmol/L acrydite tau_Fac primer, 0.1% Tween-20, 0.2% bovine serum albumin and a desired amount of cDNA template), and a final concentration of 0.087% of both ammonium persulfate and TEMED was added. We poured a 15 μm thick gel on a Bind-silane (Amersham, Pittsburgh, PA, USA) treated, glass microscope slide, and the gel was casted within a Teflon surrounded oval space (Erie Scientific, Portsmouth, NH, USA). The slides were washed 30 min in dH_20, then dried under the hood (AirClean) for ~20 min. 20 μL of PCR reagent mix containing 1x Jumpstart Taq buffer 0.2% bovine serum albumin, 0.1% Tween-20, 0.5 μL unmodified amplification primer (tau_Rev), 200 μmol/L deoxynucleotides (dNTPs), 0.335 U jumpstart Taq was poured on the gel which was covered with a coverslip and a SecureSeal chamber with mineral oil. The slides were cycled as follows: after an incubation at 94°C for 3 min, steps of 94°C for 45 s, 58°C for 30 s for the initial annealing cycle (in each subsequent cycle the annealing temperature was decreased by 0.2°C), and then 72°C for 180 s for polymerization. This was repeated for 43 cycles and the last cycle was followed by an incubation at 72°C for 6 min, then 4°C.

Hybridization and single base-extensions

Before probing, the *polony* slides were incubated in pre-warmed denaturing buffer (70% formamide, 1x saline sodium citrate buffer) at 70°C for 15 min, and washed twice (4 min each) in wash buffer 1E (10 mmol/L Tris–HCl pH 7.5, 50 mmol/L KCl, 2 mmol/L EDTA, and 0.01% Triton X-100). A frame seal chamber (MJ Research) was attached to each slide, and 60 μL of annealing mix (0.5 μmol/L sequencing primer, 6x SSPE, 0.01% Triton X-100) was added over the gel. The slides were heated at 94°C for 6 min, followed by 60°C for 15 min. Free primer was removed by washing the slides twice (4 min each) in wash buffer 1E. For single base extension, slides were then equilibrated with Klenow extension buffer (50 mmol/L Tris–HCl pH 7.3, 5 mmol/L $MgCl_2$, and 0.01% Triton X-100). Extension reactions were initiated by addition of fluorescence-labeled dNTPs, Klenow and single strand binding protein. Slides were held at 25°C for 2 min to complete the reaction. After washing, slides were scanned and the next cycle was performed.

Quantitation of the polonies

All images of gels were acquired on a ScanArray 5000 instrument (Perkin Elmer, Waltham, MA, USA) at 10 μm resolution using either a 635 nm (Cy5 detection) or 532 nm (Cy3 detection) laser. Gels were scanned after single base extensions and/or hybridization with labeled probes, as well as after post-probe stripping to assess background signal. Sixteen-bit values (1–65 535) are retained per pixel. The tau probed *polony* slide images were aligned with Adobe Photoshop 7.0 to generate a composite. Colocalization of polonies allows us to detect the isoform of the original molecule. A grid was overlaid on the images and a systematic random approach was performed to select fields for analysis. A total of 250–2500 polonies per case were counted in this fashion to provide estimates of expression. For each case, there were at least three slides created and counted. All counts were performed without knowledge of diagnosis. Initial count-recount experiments suggested that the exon distributions were reproduced within ∼5%.

Haplotyping of cases

The PCR amplification/detection of the H1/H2 genotyping of the intronic 10 deletion was as previously described (Conrad *et al.* 2004) except, the PCR primers were modified. The forward and the reverse primers were INDEL F (GGTGAGGAAGACGTTCTC-ACT) and R (CCGCTTTGTGGGGAAGACAAA). Briefly, the sequence was amplified from using the 'touchdown' PCR method (Hecker and Roux 1996). PCR was performed in a 50 μL reaction mixture containing 20 mmol/L Tris–HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L each primer, 0.2 mmol/L dNTPs, and 1 U of Taq polymerase (Invitrogen). The touchdown PCR method was as follows: after an incubation at 94°C for 3 min, steps of 94°C for 30 s, 65°C for 30 s for the initial annealing cycle (in each subsequent cycle the annealing temperature was decreased by 0.2°C), and then 72°C for 55 s for polymerization. This was repeated for 25 cycles. Finally, an additional set of 10 cycles were performed consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 55 s; the last cycle was followed by an incubation at 72°C for 55 s.

Western blot analysis

The Massachusetts Alzheimer Disease Research Center Brain Bank provided temporal cortex from 20 AD (78.4 ± 7.1 years) and 20 age-matched control (81.2 ± 8.2 years) subjects, the pathologists attempted to match the cases and adjacent brain sections from which the *polony* was performed. The diagnosis of AD was established pathologically using Consortium to Establish a Registry for Alzheimer's disease standards and Braak staging (Ingelsson *et al.* 2004; Bertram *et al.* 2005). The mean post-mortem interval for AD and controls was not statistically significantly different. In addition, dementia with Lewy bodies ($n = 8$) and control subjects ($n = 8$) were in included and were previously described (Klucken *et al.* 2006). Temporal cortex brain tissue was homogenized in 1 : 10 weight per volume of lysis buffer (10 mmol/L NaF, 1 mmol/L $NaVO_4$, 2 mmol/L EGTA, 0.2% sodium dodecyl sulfate, 1X phosphate-buffered saline, and Roche Complete protease inhibitor; Indianapolis, IN, USA). Twenty micrograms of protein was loaded onto 10–20% Novex Tris–glycine gels and transferred to polyvinylidene difluoride membranes. Htra2β1 (Stoilov *et al.* 2004) and actin were visualized and quantified using the Odyssey IR system (LICOR, Lincoln, NE, USA). Unpaired *t*-test with Welch correction determined the two-tailed $p = 0.0035$ (GraphPad Software, San Diego, CA, USA).

Results

Investigation of tau exon splicing in AD

To validate the *polony* technique for detecting altered tau splicing in clinical specimens, two FTD samples with a known tau splicing mutation were first examined. The mutation, which locates at the +3 position in exon 10, has been shown to promote exon 10 inclusion both *in vivo* (Spillantini *et al.* 1998) and *in vitro* (D'Souza *et al.* 1999). With the *polony* technique, we amplified whole tau cDNA transcripts (exons 1–11) as previously described (Zhu *et al.* 2003) and could identify all six tau isoforms. As expected, expression of exon 3 was dependent on expression of exon 2 (Andreadis *et al.* 1995). As a positive control for the ability of the *polony* assay to detect quantitative changes, we examined cDNA from two cases FTD with known exon 10+3 mutations. In these cases, the ratio between exon 10+/− was increased by 2.3-fold as compared with the control cases. This result agreed with previous estimation by quantitative PCR, further validating the *polony* platform.

We next examined tau expression profile in temporal neocortical brain tissues from 22 AD and 20 normal subjects. Each sample was evaluated at least in triplicate, and overall, more than 60 000 tau transcripts were analyzed (33 888 for AD and 32 884 for control). The large number of molecules analyzed allows us to track < 0.02% changes between two populations determined by computer simulation as previously described (Zhu *et al.* 2003). Because each of the six isoforms can be unambiguously determined, the proportion of each isoform in the tau transcriptome can be precisely determined. The pattern of tau splicing differed between AD and control cases ($p = 0.0157$) by multivariate analysis (SAS System, Cary, NC, USA). In terms of contributing isoforms, using the Student's *t*-test, the −2−3+10 variant (4R/0N, 1.4-fold, and $p = 0.0040$) was significantly higher, while the isoforms +2+3−10 (3R/2N, 1.6-fold, and $p = 0.0089$) and +2−3−10 (3R/1N, 1.48-fold, and $p = 0.0073$) were lower in AD (Fig. 2a). When individual tau exons were considered, exon 10 is higher (1.3-fold, $p = 0.0399$), exon 2 is lower (1.6-fold, $p = 0.0036$), and exon 3 is unchanged in AD when compared with normal cases (Fig. 2b). Both the exon and isoform analyses suggested a coordination between the up-regulation of exon 10 and the down-regulation of exon 2, as the differences were significant only for those isoforms (e.g. +2−3−10 but not −2−3−10) that are compatible with opposite trends in inclusion of exons 2 and 10. The observed changes are modest and likely to be missed by conventional RT-PCR or hybridization-based methods, implying that tau

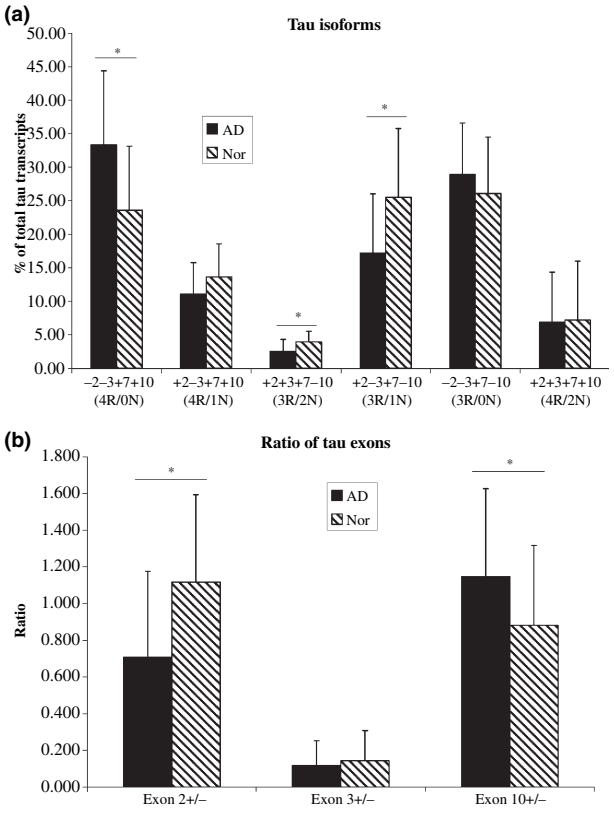

Fig. 2 Tau isoform and exon expression. (a) The mean percentage of total tau isoforms are shown for the Alzheimer's disease (AD) (n = 22) (black) and control (n = 20) (striped) subjects. (b) The mean ratio of present (+)/absent (–) a particular exon of the tau isoforms are shown. The statistically significant differences (SAS system) have a line and an asterisk above them. The data are shown with SD plus error bars.

alternative splicing is tightly regulated for the purpose of maintaining normal neuronal function.

Discriminatory analysis

The tau splicing profiles were further analyzed for individual subjects to identify a potential signature that could discriminate AD and normal cases (Fig. 3). The exon 2+/− ratio, the most significant alteration between AD and control subjects, was tested as a disease classifier. Scatter plot showed that AD and control samples are clearly separated into two clusters. While the exon 2−/+ is around 1 : 1 in most control subjects, a significantly higher ratio was observed for the AD subjects (Fig. 3). Linear discriminatory analysis showed that 64% (14/22) of the AD and 80% (16/20) of the normal subjects could be correctly assigned on the basis of exon 2 inclusion/exclusion. Using cross-validation or leave-one-out analysis, we found that the prediction error rate (PER) is 28.6%. A similar result (PER = 33%) was obtained with the ratio of tau variants suggested by reciprocal inclusion/skipping of exons 2 and 10: (−2−3+10)/[(+2−3−10) + (+2+3−10)]. Taken together, our results suggest that the

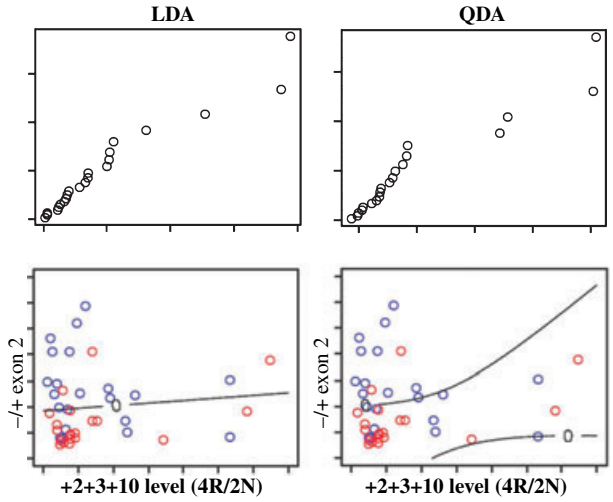

Fig. 3 Linear (LDA) and quadratic (QDA) discriminant analysis. Ratio of exon 2 absence (–)/presence (+) versus percent of the tau isoform (+2+3+10) in Alzheimer's disease (AD) (blue) and control (red) subjects.

coordinated change in tau alternative splicing may be an important signature of AD.

Analysis of tau splicing relationship with clinical and pathology profiles

A number of these AD patients (n = 14) have previously been evaluated clinically and neuropathologically (Ingelsson *et al.* 2004; Bertram *et al.* 2005). In an exploratory analysis of these AD cases, the number of neurofibrillary tangles/cubic millimeter (mm^3), amyloid level, the onset age of AD, and duration of the disease showed no significant correlation with any tau isoform and exon ratio. These results suggest that these AD-related characteristics and the degree of detected aberrant tau splicing are not tightly linked (Spillantini *et al.* 1998; D'Souza *et al.* 1999).

Evaluation of *cis* and *trans* mechanisms

The molecular basis for the splicing difference could be explained by a *cis*-acting (e.g. splice-site polymorphisms) and/or a *trans*-acting mechanism (e.g. splicing factors). There are suggestive evidences for both (Buee *et al.* 2000; Zhu *et al.* 2003; Conrad *et al.* 2004; Myers *et al.* 2005; Wang *et al.* 2005). Tau genetic studies have identified two sets of segregating polymorphisms referred as H1 and H2 clades (Baker *et al.* 1999). It has been suggested that either a subset of H1 (Myers *et al.* 2005; Laws *et al.* 2006; Myers *et al.* 2007, Mukherjee, 2007) or H2 (Conrad *et al.* 2004) is over-represented in late-onset AD. The tau haplotype was therefore determined for each patient in our cohort.

The single-base resolution of the *polony* platform prompted us to examine allelic bias of tau expression in H1/H2 heterozygous individuals (Zhu *et al.* 2003). A

coding SNP in exon 9 (N255N) was queried using the same slides from the exon-profiling experiment. Of the 40 subjects, 13 were heterozygous for H1/H2. When this subset of subjects were analyzed, they displayed an equal tau and splice variant pattern expression from each H1 or H2 allele regardless of whether they are AD or control, as previously reported (Caffrey *et al.* 2006). Exploratory analyses were then carried out to look for an association of tau splicing with H1 or H2 haplotype. In the AD and control samples, the data showed no clear dosage effects on either alternatively spliced isoforms or exons, providing evidence against a causal relationship of the H1 and H2 tau haplotypes with tau splicing or *cis* mechanism.

A potential clue to a *trans* regulatory mechanism of tau splicing has recently been reported. The splicing factor, htra2β1, in concert with other factors, can inhibit exon 2 splicing while promoting exon 10 inclusion (Wang *et al.* 2005). This concurs with our observation in AD, where the splicing of exons 2 and 10 is regulated in opposite directions. The level of htra2β1 protein was then examined (Fig. 4) and it significantly differs in AD when compared with control subjects ($p = 0.0071$, Mann–Whitney). Surprisingly, a decrease in htra2β1 level was observed in AD patients who show an increase in exon 2 skipping and exon 10 inclusion. The trend of htra2β1 alteration is opposite to the aforementioned minigene and transfection assay (Wang *et al.* 2005), indicating that the activity of htra2β1 on tau pre-mRNA might be influenced differently by context (endogenous vs. a minigene) or be cell-type dependent. To further investigate whether the alteration in htra2β1 expression is AD specific or general to other neurodegenerative diseases, we examined a non-tau-related disorder, dementia with Lewy bodies (Klucken *et al.* 2003). No difference in htra2β1 was detected in disease versus control samples (data not shown). Taken together, these findings suggest an AD-specific alteration of htra2β1, making it a good candidate for future detailed mechanistic study of skewed tau splicing *in vivo*.

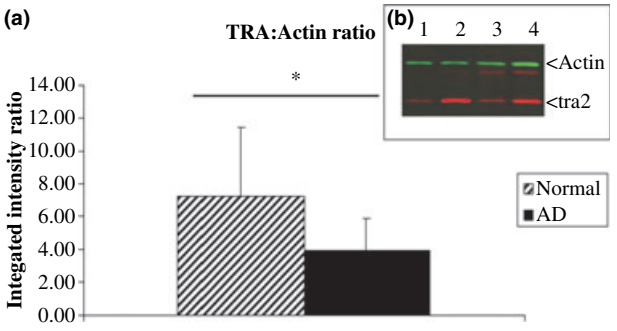

Fig. 4 Western blot analysis of normal (NC) and Alzheimer's disease (AD) subjects. (a) Graph of the data from immunoblots of brain homogenates from AD ($n = 20$) and normal ($n = 20$) subjects with actin monoclonal antibodies (AC40, Sigma) and rabbit anti-Tra2beta-1 antibodies [previously described (Stoilov *et al.* 2004)]. (b) Representative western is shown. The bands corresponding to actin (green) and Tra2 beta-1 (red) are labeled for lanes with samples from AD (lanes 1 and 3) or control subjects (lanes 2 and 4).

Discussion

Combinatorial alternative splicing is an effective way to expand metazoan proteome diversity (Maniatis and Tasic 2002). We present evidence that a loss of coordination in two alternatively spliced tau exons likely contributes to tau-related pathophysiology in AD. Our data suggest that the AD brain contains subtle changes in combinatorial tau splicing that are in some ways analogous to the elevated exon 10 levels seen in other tauopathies like FTD and progressive supranuclear palsy (Buee *et al.* 2000). In AD, we found an increase in the inclusion of exon 10 and the exclusion of exon 2 containing tau isoforms [e.g. up-regulation −2−3+10 (4R/0N) along with a decrease of both +2+3−10 (3R/2N) and +2−3−10 (3R/1N)]. Moreover, in contrast to inherited frontotemporal-dementia-tau mutations which lead to aberrant exon 10 inclusion, AD has no reported tau mutations and our current results implicate a non-genetic or *trans*-based mechanism for the alteration of tau splicing. The role of exon 10 which encodes for an additional microtubule binding repeat (4R tau) and presumably playing a role in tau–microtubule interactions was well established (Buee *et al.* 2000). Exons 2 and 3 encode for N-terminal inserts whose protein domains have been suggested to play a role in microtubule spacing in axons and also allowing for the interaction of tau with the plasma membrane (Buee *et al.* 2000); however, their role in disease along with 4R tau encoded by exon 10 remains to be elucidated.

As the alternative splicing of the tau gene in AD has been previously examined with inconclusive results (Goedert *et al.* 1989a,b; Chambers *et al.* 1999; Yasojima *et al.* 1999; Boutajangout *et al.* 2004; Umeda *et al.* 2004; Connell *et al.* 2005; Glatz *et al.* 2006; Ginsberg *et al.* 2006; Ingelsson *et al.* 2006), we attempted to verify the most robust result of increased exon 2 skipping in AD by the complementary method of quantitative PCR (QPCR). Initial experiments were performed to determine the sensitivity of the QPCR assay by amplifying full-length tau transcript or its exon 2 from mixed clones with and without exon 2. Our QPCR-sensitivity range was not enough to detect a 36% difference of exon 2 under these ideal conditions which is in line with Gentle *et al.* (2001), who reported that for QPCR techniques, as a result of assay variability, the theoretical difference detection limit is 23%. We believe that the key issue is the *polony*'s unique ability to not only detect and quantitate all six splice forms individually and simultaneously with built-in normalization as in microarrays (Ginsberg *et al.* 2006), but without traditional PCR/amplification-based biases of other approaches (Goedert *et al.* 1989a,b; Chambers *et al.* 1999; Yasojima *et al.* 1999; Boutajangout *et al.* 2004; Umeda *et al.*

2004; Connell *et al.* 2005; Glatz *et al.* 2006; Ginsberg *et al.* 2006; Ingelsson *et al.* 2006). Taken together, the size of difference detected by the *polony* technique may not be evident using QPCR approaches that focus on a single exon and normalization to a housekeeping gene. Indeed, our experience with QPCR approaches does not detect a difference in exon 10 spicing in AD (Ingelsson *et al.* 2006), similar to other reports (Goedert *et al.* 1989a,b; Chambers *et al.* 1999; Boutajangout *et al.* 2004; Umeda *et al.* 2004; Connell *et al.* 2005; Ingelsson *et al.* 2006). However, some groups have found tau 4R : 3R ratio differences in AD through the use of semiquantitative PCR [50–75% (Glatz *et al.* 2006) and 340% (Yasojima *et al.* 1999) difference, both exceed the theoretical 23% limit] or single cell microarray, a non-QPCR-based analysis [20–30% difference, similar to the *polony* detection limit (Ginsberg *et al.* 2006)]. All of the positive studies show an elevation of 4R : 3R ratio, a conclusive study could involve a comparison of precision, accuracy, and sensitivity of all of these approaches (e.g. QPCR, *polony*, and microarray) on a set of common DNA standards and subject samples.

Neuronal loss and glial proliferation in AD (Unger 1998) raise the question of what cell types contribute to the splicing alteration we observed. The tau gene is primarily expressed in neurons; however, it is also found to a lower extent in other CNS cells, oligodendrocytes (LoPresti *et al.* 1995; Muller *et al.* 1997) and developmentally, in astrocytes (Couchie *et al.* 1988). In our subjects, the expression of a glial cell marker, glial fibrillary acidic protein (GFAP), was found to be increased in AD (Ingelsson *et al.* 2006). We asked whether tau splicing patterns correlated with the degree of GFAP increase by using the Kendall ranking test for comparing the rank orders of the cases for GFAP levels by QPCR (Ingelsson *et al.* 2006) and with the present results of either exon 2 or 3 or 10 levels. Our analysis only detected an inverse correlation between the levels of exon 3 and GFAP, as might be expected with neuronal loss and gliosis. Furthermore, Nishimura *et al.* (1997) report a lack of exon 3 in glia in the CNS thereby providing evidence that exon 3 is found predominately in neurons. As exon 3 is unchanged in AD when compared with control cases, these results argue against a shift in glial/neuronal ratios as the source of the alteration of tau splicing in AD. As our results represent an average across a variety of CNS cell types with and without pathology, laser capture microscopy (LCM) of neurons (Schindler *et al.* 2005) coupled to the *polony* would help to investigate origin of splicing differences further. Ginsberg *et al.* (2006), using single cell aspiration, a LCM analogous technique, of neurons from AD and microarrays have reported an exon 10 shift when compared with control, while Ingelsson *et al.* (2006) using LCM of neurons and QPCR observed no changes of tau splicing, suggesting further analysis is required possibly comparing all approaches from common LCM-generated samples.

Our data suggest that there is a unique pattern of tau exon 2 exclusion/tau exon 10 inclusion in AD compared with control brains. The tau protein isoforms in AD that have been examined so far show no clear difference between AD and controls (Fujino *et al.* 2005; Ingelsson *et al.* 2006; de Silva *et al.* 2006). These results are in contrast to FTD-17, in which significantly altered exon 10 mRNA splicing leading to increased four-repeat tau protein are detectable. The skewing of exon 10/increased 4R tau protein in FTD-17 is believed to be the major defect, indicating that an underlying mechanism may be shared but to different extents in AD, FTD-17 and other distinct neurodegenerative diseases. Interestingly, a tau exon 2 splicing defect was also observed in myotonic dystrophy type I, a multisystemic disorder characterized by expanded CTG repeats and tau aggregates (Sergeant *et al.* 2001), suggesting AD and dystrophy type I may share a tau pathology mechanism as well.

The contributions of *cis* versus *trans* mechanisms for altered tau splicing in AD were explored. Although Myers *et al.* (2007) showed that subtypes of the H1 halpotype clade might carry *cis* elements responsible for both tau expression and splicing, our findings suggest that an AD specific alteration in htra2β1 expression as a potential mechanism for altered tau splicing. Recently, an over-expression of htra2β1 has been demonstrated to facilitate the inhibition of exon 2 splicing and promoting exon 10 inclusion *in vitro* (Wang *et al.* 2005). However, we observed a decrease of htra2β1 protein and an increase of exon 2 exclusion/exon 10 inclusion in AD brain when compared with controls. In addition, Glatz *et al.* (2006) have reported an elevation of htra2β1 mRNA in AD who suggests that the normal regulation of htra2β1 level is disrupted. Cell biology studies of the endogenous htra2β1 protein showed that in the context of other splicing factors can coordinate the alternative splicing of several genes including htra2β1 itself. As htra2β1 protein increases, a shift in favor of the alternative splicing of the htra2β1 transcript leads to the generation of a truncated non-functional protein, htra2β3, creating a negative feedback loop (Stoilov *et al.* 2004). Taken together, the autoregulation of htra2β1 is altered, possibly through a post-translational down-regulation of htra2β1 protein (e.g. half-life) in the AD brain, suggesting that htra2β1 is an attractive target to understand the pattern of tau splicing in normal and disease biology.

As splicing is implicated in many tauopathies (Buee *et al.* 2000), further investigation with new technologies like the *polony* technique, along with systematic evaluation of splicing factors, (e.g. htra2β1) would enhance the ability to monitor possible splicing dysregulations and to guide rational therapeutic designs in these related but distinct diseases. Recently, Riessland *et al.* (2006) showed that a histone deacetylase inhibitor (M344) can up-regulate htra2β1 expression several fold and correct a splicing imbalance in the genes causing spinal muscular atrophy. Although not

examined, it is likely that *in vitro* and *in vivo* tau splicing could also be affected by this same treatment.

Similar to tau, many disease-related genes are subjected to combinatorial alternative splicing that might be altered to various extents in the disease states and in the normal biology of different brain regions thereby investigating natural splicing heterogeneity as important follow-up experiments to our study as well. Knowledge of their splicing profiles may require single-molecule-based technologies like the *polony*, by which the *cis*-relationship of multiple alternatively spliced exons can be precisely determined. Although powerful for profiling a single-gene transcriptome, the *polony* platform is limited by its current throughput from genome-wide application. However, a variation of the *polony* technique using the high-throughput parallel sequencing technology (Shendure *et al.* 2005) could provide an avenue towards comprehensive profiling of alternatively spliced mammalian transcriptomes (Buhler *et al.* 2004).

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