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Non-phosphorylated tau protein in the CSF as a novel potential biomarker of Alzheimer's disease: Analytical and diagnostic validation

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Abstract

BACKGROUND: Tau protein, along with its phosphorylated forms (pTau), is one of the established cerebrospinal fluid (CSF) biomarkers of Alzheimer's disease (AD), but virtually nothing is known about a potential diagnostic role of non-phosphorylated tau molecules (Non-P-Tau) in CSF.

OBJECTIVE: To establish, and analytically and clinically validate the first assay capable to measure concentrations of Non-P-Tau in human CSF.

METHODS: An antibody (1G2) was developed that selectively binds to the non-phosphorylated tau molecule at positions 175, 181, and 231, and was used for establishing an ELISA capable to measure Non-P-Tau in human CSF. In the analytical validation, linearity, repeatability of the standard curves, and intra- and interassay precision of the assay were tested, as well as inter-center variability with the QC samples sent either frozen or under ambient temperature. In the clinical part, concentrations of Non-P-Tau were measured in CSF samples from carefully selected AD or mild cognitive impairment (MCI) patients, whose diagnoses were supported by the results of the "classic" CSF biomarkers (n=58), and as well as in CSF samples from non-demented controls (n=42).

RESULTS: The 1G2 antibody reacts with decreasing reactivity to tau peptides containing phosphorylation at positions T175, T181 and T231. The average OD of the blank sample was 0.067 ± 0.006; the CV's of the optical densities of the repeated standard curves were between 3.6 - 15.9%. Median intra-assay range-to-average imprecision of double measurements was 4.8%; inter-assay imprecision of was in the range of 11.2% - 15.3%. Non-P-Tau concentrations are stabile in the CSF samples sent to distinct laboratories under ambient temperature; inter-laboratory variation was approximately 30%. The Non-P-Tau CSF concentrations were highly significantly increased in the AD/MCI group (109.2 ± 32.0 pg/mL) compared to the Controls (62.1 ± 9.3 pg/mL, $p<0.001$). At the cut-off of 78.3 mg/mL, the sensitivity and the specificity were 94.8% and 97.6%, respectively.

CONCLUSIONS: For the first time, an assay is reported to reliably measure CSF concentrations of non-phosphorylated tau.

Introduction

A crucial role of the cerebrospinal fluid (CSF) biomarkers in an early diagnosis of Alzheimer's disease (AD) has been extensively discussed, leading to inclusion of the Neurochemical Dementia Diagnostics (NDD) biomarkers into different diagnostic and/or research criteria [1-3] + Dubois-IWG. Such development is not surprising when considering the need for a reliable early AD diagnosis in clinical trials and practice. Research on medical interventions in AD focuses on the early stages of the disorder, for example mild cognitive impairment (MCI), and hence CSF biomarkers would be helpful to identify individuals in the pre-dementia phase of AD [4]. This was also reflected in the European Medicines Agency (EMA) statement that AD CSF biomarkers are useful for the enrichment of the prodromal AD populations in clinical trials [5].

Alterations in the CSF occur many years or even decades before the onset of the clinical symptoms of AD [6, 7]. Currently two groups of molecules in the CSF are accepted as NDD biomarkers: amyloid β (Aβ) peptides, which reflect deposition of Aβ (senile) plaques in the brain, and tau protein along with its hyperphosphorylated forms (pTau), which is linked to the accumulation of neurofibrillary tangles and neurodegeneration (reviewed in). Although alterations in Aβ metabolism are currently considered the earliest detectable events in AD [6], intervention strategies based on the Aβ hypothesis have been so far disappointing [8, 9]. This calls for more extensive investigation of other hypotheses, of which those related to tau seem particularly attractive [10]. This is further supported by the observation that cognitive symptoms in AD are directly related to biomarkers of neurodegeneration rather than biomarkers of Aβ deposition (reviewed in [6]); also in neuropathologic studies a clear correlation was shown between the degree of neurofibrillary tangle pathology post mortem and a patient's cognitive functions intra vitam [11, 12].

Whereas the diagnostic role of tau and its phosphorylated forms, in particular those phosphorylated at threonine 181 (pTau181), has been extensively studied in the last decade, we are not aware of any study addressing the diagnostic role of CSF non-phosphorylated tau epitopes (Non-P-Tau); furthermore, we have not found any reports of an assay capable to specifically measure CSF concentrations of Non-P-Tau. Hence it seemed relevant to establish such an assay and to investigate whether Non-P-Tau could be interesting as a potential novel biomarker of AD.

Materials and Methods

1. Preparation of phosphorylated aggregated tau

2mg/500µl recombinant human tau (2N4R isoform 441 amino acids) was incubated for 20h with 3µg activated MAPK13 (Life Technologies, USA) in 10 mM Tris (pH 7.4), 11 mM MgCl2, 1 mM EGTA, 1 mM DTT, 1mM AEBSF, 0.5 mM Na3VO4, 0.01% TritonX-100, 1 mM ATP at 30°C. Fresh ATP was supplemented after 10h incubation. Tau aggregation was started by adding 20fold-aggregation mix (1M MOPS pH 6.5, 1M NaCl, 8mg/ml Heparin, 2mM AEBSF) after heat inactivation of MAPK13 and phosphorylated tau was incubated 48h at 37°C. Monomeric (fraction 45-47) and aggregated phosphorylated tau protein (fractions 21-23) were separated using a Sephacryl S500 gelfiltration column as described by [13].

2. Immunisation and antibody development

Fifty µg of phosphorylated and aggregated Tau441 in complete Freud adjuvant (Sigma-Aldrich, USA) were used for the first immunization of Balb/c mice by subcutaneous injection. Thereafter animals were immunized 4 x using incomplete Freud adjuvant during 56 days followed by 3 x intravenous booster injections of antigen in 0.02 M phosphate buffered saline pH 7.4 (PBS). Spleen cells were collected and fused to mouse myeloma cells X63Ag8.653 using PEG 1500 (Roche Diagnostic GmbH, Mannheim, Germany). Fused cells were selected using Hypoxanthine-Aminopterin-Thymidine (HAT) medium (Sigma-Aldrich) with Hybridoma Fusion and Cloning Supplement (HFCS) (Roche) during 14 days. Clones were selected regarding their reactivity to Tau441 and their IgG production. A clone producing monoclonal antibody 1G2 could be selected and were re-cloned twice using the limiting dilution method. The antibody was tested for reactivity to tau isoforms (rPeptide, USA) in Western Blot, for reactivity to tau and tau fragments in ELISA and Western Blot and to tau peptides not phosphorylated and phosphorylated at amino acids 175, 181, 199, 202, 231 and 235 of Tau441 in ELISA. Inhibition experiments using peptides of Tau441 containing amino acids 175, 181 and 231 not phosphorylated and phosphorylated were performed on Tau441 coated ELISA plates as described by Kovacs et al. (2012). Antibody isotype was determined using mouse immunoglobulin isotyping strip test (Roche, Suisse). Epitope mapping was performed using 12 amino acid peptides of Tau441 as described by [14].

3. Development of Sandwich-ELISA for detection of none phosphorylated Tau

Maxisorb®plates (Thermo Scientific, USA) were coated with 5µg/ml 1G2 antibody in 0.05 mol/l Na2CO3/NaHCO3, pH 9.6 with 120 µl per well at 4-8°C overnight. Coated plates were aspirated and blocked using 50 mM Tris buffer pH 7.5 containing 0.15 M NaCl and 0.05% Tween 20 (washing buffer) containing 3 % BSA fraction V (Serva, Heidelberg, Germany). Using different concentrations of Tau441 (rPeptide) lyophilized standards and controls were designed by means of dilution buffers containing BSA fraction V (Serva) and skim milk powder (Carl Roth, Germany) basing on phosphate buffered saline containing Tween 20 as detergent. The assay was developed studying different incubation protocols and 3 h room temperature on orbital shaker was found appropriate for antigen binding as first step. Washing was performed 5 times using 50 mM Tris buffer pH 7.5 containing 0.15 M NaCl and 0.05% Tween 20 and followed by 90 min incubation with HRP conjugated detection antibody 7E5 (Analytik Jena AG, Germany) at room temperature that binds Tau441 between amino acids 156-165. After additionally 5 times washing TMB staining was performed by means of ready to use staining solution for 30 min in the

dark followed by termination with 1.5 M H_2SO_4. Optical density was measured at 450nm as well as 450/620 nm.

4. Determination of the intra- and inter-assay imprecision; repeatability of the standard curves

Intra-assay imprecision was calculated from the duplicate analyses of one hundred CSF samples, and expressed as median of the range-to-average of the duplicates.

Inter-assay imprecision was determined with three quality control (QC) samples prepared from the pooled human CSF samples. Briefly, CSF pools were stored at -80°C, and thawed once for the preparation of the three QC samples (coded QC1, QC2, and QC3) for this study. After the preparation, aliquots were re-frozen and kept at -80°C. Immediately before the analysis, one set of the three QC samples was freshly thawed. The samples were assayed on eight ELISA plates on eight different days, by the same operator. The readings of the optical densities (OD's) were performed in two modes: (A) without the reference wavelength (i. e. measurement at 450 nm only) and (B) with the subtraction of the OD's obtained at the reference wavelength (620 nm). The imprecision for each QC sample is expressed as the percentage coefficients of variation (CV's) defined as the standard deviation divided by the average of eight measurements.

To test repeatability of the standard curves, six independent runs were performed on six different days.

5. Inter-center quality control

For the inter-center study, four pairs of pooled CSF samples were prepared; from each pair, each of the nine participating centers received one aliquot frozen on dry ice and one aliquot shipped at ambient temperature. The latter had to be deep frozen upon arrival and kept at -80°C until the analyses. The participating centers were informed that they would receive eight samples (four frozen and four in liquid status) but not that the frozen and liquid samples are matched.

6. Clinical validation: Patients and samples handling

The study on the human samples was approved by the ethical committee of the University of Erlangen-Nürnberg. All patients, or their close relatives, gave their written informed consents. For the clinical validation, the analyses were performed in the CSF samples from very carefully selected and characterized patients with Alzheimer's Disease (AD) or Mild Cognitive Impairment with AD pathology (MCI-AD) (Positive Group, n=58) and Non Demented Controls (Control Group, n=42). AD/MCI patients were diagnosed and sub-classified according to the current recommendations from the NIA-AA working groups [1, 2], including analyses of the "classic" four AD biomarkers: Aβ1-42, Aβ42/40 ratio, Tau, and pTau181. The characteristics of the groups are presented in table 1. The samples were collected by lumbar puncture (LP) into polypropylene test tubes according to a protocol described elsewhere [15], centrifuged, aliquoted, and stored at -80°C until the analyses.

7. Statistical analysis

If not stated otherwise, the results are presented as averages ± standard deviations (SD's). Imprecision is reported as coefficients of variation (CV's) or as ranges-to-averages (in case of duplicate determinations). The area under the receiver operating characteristic (ROC) curve is reported with the corresponding 95% confidence interval (95% CI). Statistical comparison of the patient groups was done with the Mann-Whitney test. The cut off for the separation of the patient groups, and the corresponding sensitivity and the specificity, were calculated at the maximized Youden Index. The analyses were performed with Statistica and MedCalc. A $p<0.05$ was considered significant.

Results

1. Analytical selectivity; intra- and inter-assay imprecision

Figure 1 presents schematically the tau molecule with the epitopes addressed in this study as well as the corresponding kinases known to phosphorylate them [16-18].

Antibody 1G2 was determined as IgG2b isotype. Epitope mapping (Fig. 2) shows binding sites of 1G2 to peptide sequences KTTP (174-177), KTTP (180-183) and RTTP (230-233). The antibody reacts in manner of decreasing reactivity to tau peptides containing phosphorylations at positions T175, T181 and T231 (Fig. 3a). Binding of 1G2 to Tau441 coated ELISA plates (100 ng/ml) was most decreased by adding tau peptides containing all three binding sites whereas each binding site alone could not inhibit binding of antibody to Tau441 (Fig. 3b).

The median intra-assay range-to-average imprecision of one hundred double measurements was 4.8% (interquartile range 2.4 - 8.9%). Among these duplicate determinations, none resulted in the imprecision exceeding 20%, which normally would lead to a repetition of the measurement in the everyday diagnostic routine. The results of the inter-assay imprecision are presented in the table 2. The comparison of the two reading modes (with- and without wavelength correction) shows clearly better precision when the optical density is read out without correction; correspondingly, we used the non-corrected reading mode for all measurements in this study.

2. Standard curves

The average OD of the blank sample was 0.067 ± 0.006. The plots of the OD's versus the defined concentrations of the standards are presented in the figure 4. The goodness of fit of the average standard curve was > 0.99. The CV's of the optical densities obtained in six repetitions of the standard curves were between 3.6 - 15.9%.

3. Inter-center comparison

The results of the inter-center comparison (averages of the reported concentrations and the corresponding coefficients of variation) are presented in the table 3 and, exemplarily for two pairs of samples, in the figure 5.

4. Clinical validation

The results of the measurements of Non-P-Tau in the patients CSF samples are presented in fig. 6. The concentrations were highly significantly increased in the AD/MCI group (109.2 ± 32.0 pg/mL) compared to the Controls (62.1 ± 9.3 pg/mL, $p<0.001$, fig. 6a). At a cut-off of 78.3 mg/mL, the sensitivity and the specificity were 94.8% and 97.6%, respectively. The area under the ROC curve (fig. 6b) was 0.976 (95% CI: 0.923 to 0.996).

Discussion

In this paper, we present the development, and the analytical and clinical validation of an assay capable to specifically measure the concentrations of non-phosphorylated tau molecules in human CSF. NDD relays currently on the analysis of the CSF biomarkers belonging to two groups: amyloid β peptides and tau proteins [19]. The latter includes also phosphorylated forms, which emerged from the observation that the tau molecule is hyperphosphorylated in tau deposits in the AD brain [10, 20]. Whereas increased CSF concentration of tau is a sensitive marker of neurodegeneration, but entirely unspecific for AD, increased

concentration of pTau molecules seems much more AD specific [21, 22]. On the other hand, virtually nothing is known about the possible diagnostic utility of non-phosphorylated tau epitopes.

Due to its unfolded, highly hydrophilic nature, tau is a highly phosphorylation-prone protein with 85 (in case of the longest isoform, i. e. 2N4R) potential phosphorylation sites. In experimental conditions, about half of them have been observed to be phosphorylated (currently reviewed in [10]). Its main role is to stabilize axonal structures, whereas phosphorylation-dephosphorylation regulates directly its association and dissociation from the neuron's microtubules. Under physiologic status, tau contains on average two phosphorylated sites; this number can increase to 7-8 sites under pathologic conditions such as AD [23]; correspondingly, "phosphorylated tau" is actually a mixture of differently phosphorylated tau proteins.

By immunization with phosphorylated tau protein, we obtained the monoclonal antibody 1G2 with a specificity to the TPP sequence in its unphosphorylated form flanked by a basic amino acid at the N-terminus. This resulted in three binding sites on 2N4R Tau containing 441 amino acids. Threonine 181 and threonine 231 are two main phospho-tau positions addressed in the assays utilized for AD diagnostics, and providing a similar clinical performance [24]. Here, we chose the unphosphorylated T181 and T231 (in addition to T175) epitopes for our novel assay. Binding of 1G2 antibody to Tau441 increases by availability of free TPP motives (figure 3b) and this characterizes a very strong avidity for maximum of three free binding sites. Nevertheless, T175, T181 and T231 are phosphorylated by several kinases that are not specific for only one of these positions and it could be assumed that detection of such non-phosphorylated tau is not dependent on activity of only one unique kinase. Capturing of this non-phosphorylated tau from CSF using 1G2 as capture antibody could not be defined as specific for one of these three binding sites. pTau181 and pTau231 have been widely described as biomarkers of AD [21, 24, 25], whereas, to our best knowledge, pTau175 has not been reported so far in the context of its possible diagnostic relevance. Based on the results of our analysis we assume that this tau population bound by 1G2 antibody is rather a minor part of total tau in CSF and we can assume that strongest avidity obtained by using two binding sites on a single tau molecule could be a requirement for detection of such low concentrations.

Analytical validation of the assay revealed its very good performance, with reasonable intra- and inter-assay precision, and standard curve repetition, comparable to the parameters observed in other NDD assays [26]. Noteworthy is that among duplicate determinations of one hundred patients' samples, none resulted in the imprecision exceeding 20%, which would force repetition of the measurement in the everyday diagnostic routine. The inter-center study with the paired frozen/non-frozen QC samples indicated no differences between the two preanalytical sample handling and shipping procedures: in virtually all cases the results obtained from a frozen sample were practically identical, in terms of the measured concentrations as well as the imprecision, as the results obtained from a matched sample shipped under ambient temperature. We believe this has important practical implication, since patients samples can be shipped to a distant laboratory at ambient temperature and without freezing, which significantly decreases costs of shipment, at least as long as a sample is delivered within 4-5 days. Furthermore, QC samples can be similarly shipped at ambient condition (for example, in the kit). On the other hand, similarly to other CSF biomarkers routinely used in AD diagnostics, Non-P-Tau measurements characterize currently with relatively high inter-center variability (approximately 30%) [27, 28]. This situation definitely should be improved; however, before successful measures are undertaken to reduce inter-center variability, establishing center-specific reference ranges for diagnostic purposes could help solve the problem of diagnostic-relevant interpretation.

To properly interpret the clinical validation results, particularly the ROC curve, Youden Index, and the corresponding sensitivity and specificity, it has to be taken into consideration that the NDD biomarkers, including Tau and pTau181, were used to classify the patients. This significantly improves the categorization of the groups [3, 29]; however, it has a drawback that it biases the estimation of the overall diagnostic value of a novel candidate biomarker.

We are aware of at least two limitations of our study: (a) in our clinical part, we included only patients with AD and non-demented controls. Certainly it would be interested to see if Non-P-Tau could

be helpful in differential diagnostics of other dementing conditions, particularly tauopathies (for example, fronto-temporal lobar degeneration). (b) as already stated, our strategy of patient inclusion relies on the application of "classic" NDD biomarkers to support clinical and neuropsychological diagnoses. Therefore, a further study with non-NDD biased subjects, whose diagnoses are, for example, confirmed by neuropathology, is certainly needed.

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Figure legends

Fig. 1. Schematic presentation of the Tau molecule, the three epitopes addressed in this study, and the corresponding kinases.

Fig. 2. Epitope mapping of 1G2 antibody on 12 amino acid peptides of 2N4R TAU441 with 10 amino acid overlapping. Peptides are spotted on NC membranes 10 amino acids overlapped. Membranes blocked with 5% skim milk powder in Tris buffer pH 10 containing 0,1 % Tween 20. After 1 µg/ml 1G2 was incubated in blocking buffer at room temperature overnight followed by 3 x washing using Tris buffer and anti-mouse IgG antibody HRP conjugated. Staining was performed using Western blot staining solution based on TMB. Antibody 1G2 binds to peptide sequences containing KTTP or RTTP motiv. Spots of these 12 amino acid peptides showing reactivity of 1G2 are described for line 3 and line 4 of the NC membrane.

Fig. 3. Specificity of 1G2 antibodies.

3a. Binding of antibody 1G2 to Tau peptides containing different phosphorylated amino acids coated on ELISA plates. Bound 1G2 was detected using anti-mouse-IgG antibody HRP conjugted followed by TMB staining.

3b. Inhibition of Tau441 capturing by 1G2 antibody by competition with different peptides containing phosphorylations on 181 and 231 positions. Sandwich ELISA with 1G2 coated on ELISA plates, overnight incubation at 2-10°C with Tau441 and competing peptides and detection of captured antigen (Tau441) using 7E5 antibody HRP conjugated followed by TMB staining. (The figure left and right panel should contain identical names for the peptides i.e non-pTau peptide, pT175 peptide, pT175/181 peptide, pT175/181/231 peptide)

Fig. 4. Reproducibility of the standard curves of the assay; presented are average optical densities (OD's) and their standard deviations; the insert presents the zoom-in of the four lowest standards.

Fig. 5. Results of the inter-center variability study: presented are the reported concentrations of two pairs of QC samples (A/E and B/F) sent frozen (filled symbols) and under ambient temperature (open symbols).

Fig. 6. Non-P-Tau concentrations in the patients' groups, whose clinical and neuropsychological diagnoses were supported by the "classic" AD biomarkers.

6a. Concentrations in the patients' CSF (circles) with medians and inter-quartile ranges (bars). The long horizontal bar presents the cut off at the maximal Youden Index.

6b. Receiver Operating Characteristic Curve.

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