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Technical note

Gold nanoparticle-based immuno-PCR for detection of tau protein in cerebrospinal fluid

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ABSTRACT

Tau protein in cerebrospinal fluid (CSF) is an important biomarker of Alzheimer's disease and some other brain diseases. Enzyme-linked immunosorbent assays (ELISAs) have been mostly used for quantification of tau and other biomarkers in CSF. However, these assays do not have sufficient sensitivity and dynamic range. In this study we tested the suitability of gold nanoparticles functionalized with tau-specific monoclonal antibody and oligonucleotide template for immuno-polymerase chain reaction (Nano-iPCR) quantification of tau protein in human CSF samples and compared it with ELISA, either commercial or newly developed with tyramide signal amplification. Our data indicate that Nano-iPCR is superior in sensitivity and detection range to ELISA in tau protein detection.

1. Introduction

Cerebrospinal fluid (CSF) is an important source of protein biomarkers that reflect pathological changes in the brain and provide information necessary for diagnosis and treatment of various brain-associated diseases. One of the meaningful CSF biomarkers of neuronal and axonal degeneration is the tau protein. Very high levels of tau are characteristic for patients with extensive neuronal degeneration, such as Creutzfeldt–Jacob disease (Otto et al., 1997). Enhanced levels of total and phosphorylated tau associated with decreased levels of the 42 amino acid form of β-amyloid in CSF have been used for diagnosis of Alzheimer's disease (AD), the most frequent form of dementia (Dubois et al., 2007). Quantification of total tau and other CSF markers of AD is mostly done with commercial enzyme-linked immunosorbent assays (ELISAs). However, these assays are expensive and do not have the desirable dynamic range. New low-cost methods for simultaneous detection of samples with both low and high levels of tau proteins are therefore needed.

We and others have recently introduced the polymerase chain reaction (PCR) combined with sandwich ELISA-like strategy where the target antigen is immobilized by antibodies to PCR plate wells and subsequently detected by immuno-PCR using gold nanoparticles (Au-NPs) functionalized with both antigen-specific antibody and PCR oligonucleotide template. This assay, called Nano-iPCR, proved superior in detection of cytokines and viral proteins to standard ELISAs (Potůčková et al., 2011; Chen et al., 2009; Perez et al., 2011). Here we present data confirming the utility of Nano-iPCR for quantification of tau protein in CSF samples. Compared to commercial or newly

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developed ELISAs, quantification of tau protein with Nano-iPCR is superior in sensitivity and detection range.

2. Materials and methods

2.1. Materials and reagents

Purified mouse monoclonal antibody specific for tau protein, HT7 (IgG1), and its biotinylated form were purchased from Autogen Bioclear (Calne, UK; Cat. No. 90222 and 90214). The antibody recognizes an epitope located in amino acid region 159–163, according to the human full-length four-repeat tau, tau 40 (Goedert et al., 1989). Purified mouse monoclonal antibody, TAU-5 (IgG1), which recognizes an epitope located in amino acid region 210–230 in tau 40 (Carmel et al., 1996), was obtained from Abcam (Cambridge, UK; Cat. No. ab80579). The kit for determination of total tau (INNOTEST hTAU Ag) was from Innogenetics (Gent, Belgium). Recombinant human Tau-441 protein (2N4R variant) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Colloidal Au-NPs (30 nm in diameter) at a concentration of approximately 2×10^{11} particles/ml were obtained from BBInternational (Cardiff, UK). 5′-thiol-modified oligonucleotide primer 1 [5′-(5ThioMC6-D//iSp18)CCTTGAACC TGTGCCATTTGAATATATTAAGACTATACGCGGGAACA-3′], where iSp18 is an 18-atom hexa-ethyleneglycol spacer connecting the thiol reactive group and the DNA sequence, primer 2 (5′-CCTTGAACCTGTGCCATTTG-3′) and primer 3 (5′-GTCCCTCC ATCTTCCTACTGTTCCACATGTTCCCGCGTATAGTCTT-3′) were obtained from IDT (Coralville, IA, USA). Other chemical were from Sigma-Aldrich (St. Louis, MO, USA). Lumbar CSFs from 32 individuals were collected, centrifuged, aliquoted in 1 ml polypropylene tubes and stored at −80 °C in accordance with established guidelines (Teunissen et al., 2009) until analysis. All participants signed an informed consent form. The study was approved by the Ethics Committees of the University Hospital, Královské Vinohrady and Prague Psychiatric Center.

2.2. Preparation of functionalized gold nanoparticles

Au-NPs functionalized with antibodies and oligonucleotides were prepared as described previously (Hill and Mirkin, 2006; Potůčková et al., 2011) with some modifications. Briefly, 1 ml of colloidal Au-NPs (30 nm in diameter) was incubated for 30 min at room temperature with HT7 antibody at optimal concentration (10 μg/ml), determined by Au-NP-antibody loading test (Hill and Mirkin, 2006); suboptimal concentration of the antibody resulted in rapid aggregation of the particles, whereas excess antibody prevented binding of the thiolated oligonucleotides in a later step. Then, 10% Tween 20 (10 μl) and 2 M NaCl in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4; 100 μl) were added, followed by 5′-thiol-modified oligonucleotide primer 1 at final concentration 4 nmol/ml. After overnight incubation at 4 °C with gentle stirring, the samples were salted by adding 50 μl aliquots of 2 M NaCl in PBS in four 1-h steps. The armed Au-NPs were further stabilized by adding 20 μl of 10% bovine serum albumin (BSA) and incubating for 30 min at room temperature. Free oligonucleotides were removed by three centrifugation steps through discontinuous glycerol gradient (Potůčková et al., 2011); the pellet was finally resuspended in 1 ml of PBS containing 20% glycerol, 1% BSA, 0.05% Tween 20, and 0.02% NaN_3.

2.3. Nano-iPCR

Fifty microliter aliquots of capture antibody (2.5 μg/ml TAU-5) in 50 mM borate buffer (pH 9.5) were dispensed into wells of a real-time 96-well plate (Eppendorf, Hamburg, Germany). After overnight incubation at 4 °C the wells were washed (200 μl/well; four times per washing step, if not specified otherwise) with TBS (10 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing 0.05% Tween 20 (TBST) and the remaining binding sites were blocked by 6-h incubation at 4 °C with TBST supplemented with 1% BSA and 1% casein. The wells were then washed with TBST followed by addition of 50 μl serially diluted recombinant human tau protein (Tau-441; 1–100,000 pg/ml in PBS-0.1% casein), PBS-0.1% casein alone (negative control) or undiluted human CSF samples. After overnight incubation at 4 °C, the wells were washed with TBST, followed by addition of 50 μl aliquots of Au-NPs armed with thiolated DNA oligonucleotide and HT7 monoclonal antibody, diluted 1:10,000 in PBS-0.1% casein. The wells were incubated for 1 h at 37 °C, washed with 200 μl TBST and finally with 200 μl MilliQ water. Next, 50 μl aliquots of PCR master mix solution containing Taq DNA polymerase, nucleotides, SYBR green I, 1,2-propanediol, and trehalose (Horáková et al., 2011) and supplemented with 60 nM oligonucleotide primers 2 and 3 were dispensed into each well. The plates were then sealed with Light cycler 480 sealing foil (Roche, Mannheim, Germany) and the amount of template DNA bound to antigen-anchored functionalized Au-NPs was evaluated by real-time PCR using Realplex^4 Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: denaturation at 94 °C for 1 min, followed by 40 cycles at 94 °C for 20 s, 53 °C for 20 s, and 72 °C for 20 s. The samples without template DNA were used in each run as negative controls.

2.4. Sandwich ELISAs

Newly developed sandwich ELISA with tyramide signal amplification (TSA) was routinely performed in high-binding 96-well half-area plates (Costar Corning Inc., Corning, NY, USA) or TopYield Strips (Nunc, Roskilde Site, Denmark). Capture anti-tau antibody, TAU-5, was coated at a concentration of 2.5 μg/ml in borate buffer pH 9.5 (30 μl/well) by overnight incubation at 4 °C. The plates were washed with TBST and free binding sites were blocked by adding TBS with 2% BSA (TBS-2% BSA; 185 μl/well). After 6 h at room temperature the plates were washed again with TBST and incubated overnight at 4 °C with recombinant human tau standard (Tau-441) diluted in PBS-1% BSA or tested samples (30 μl/well). Similar results were obtained when the samples were diluted in PBS-0.1% casein. Wells washed with TBST were then incubated for 1 h at room temperature with biotinylated anti-tau antibody HT7 at concentration 0.5 μg/ml in TBST-1% BSA (30 μl/well). After washing, the plates were incubated for 45 min at room temperature with extravidin-peroxidase diluted 1:5000 in TBST-1% BSA (30 μl/well). Sensitivity of the assay was increased by biotinyl-tyramide signal amplification (TSA) using the ELAST ELISA Amplification System (tyramide; PerkinElmer Life Sciences, Boston) as previously described (Dráberová et al., 2013). Absorption was measured at 450 nm

with a Sunrise plate Reader (TECAN). Background of the negative control was subtracted from the determined values.

The Innotest hTAU Ag (Innogenetics, Gent, Belgium, Cat. No. 80323) commercial kit for tau protein quantification was used according to the manufacturer's directions.

2.5. Statistics

Calibration curves were constructed after plotting the quantification cycle (C_q) values or absorbance against tau concentrations using a four-parameter logistic regression model function (variable slope) and linear regression showing the regression correlation coefficient (R^2). The limit of detection (LOD) was calculated as the mean of the negative control (NC; sample diluent) plus 3× standard deviations of NC. Correlation analyses were performed using Pearson's coefficient for normally distributed and Spearman's coefficient for non-Gaussian data. Bland–Altman curves were constructed for comparison between different methods for detection of the tau protein level. Precision profiles, reproducibility, and accuracy were expressed by the intra- and inter-assay coefficients of variation (CV). Mean of individual CVs was calculated for each standard and sample as standard deviation divided by the mean. The results were then multiplied by 100 for expression as a percentage. For recovery experiments, high (4000 pg/ml), medium (800 pg/ml) and low (100 pg/ml) concentrations of recombinant tau were spiked into validated samples and analyzed in Nano-iPCR. The results are expressed as a percentage of analyte recovered. Linearity of dilution was determined in CSF samples diluted 2–16-fold and expressed as percent linearity for all samples in the dilution series.

All statistical analyses were performed using program Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

3.1. Quantification of recombinant tau protein by Nano-iPCR and comparison with ELISA

The Nano-iPCR method for quantification of tau protein is based on immobilization of the protein on TAU-5 antibody directly in PCR wells and its detection by real-time PCR with the Au-NPs functionalized with tau-specific monoclonal antibody HT7 and a single-stranded thiolated oligonucleotide. In pilot experiments, the Au-NP-antibody loading test was used to determine the optimum concentration of the HT7 antibody for Au-NP loading. From a range of HT7 antibody concentrations (1–16 μg/ml), 10 μg/ml was found optimal for antibody and oligonucleotide arming as determined by sensitivity of tau detection and real-time PCR analysis (Potůčková et al., 2011), respectively. Moreover, Au-NPs functionalized with HT7 at the optimal concentration showed the best performance during storage for up to eight months. PCR amplification is extremely sensitive to the presence of very low amounts of antibody/oligonucleotide-functionalized Au-NPs bound nonspecifically to the walls of PCR wells, which can enhance the background signal. This problem was solved by optimizing the washing and blocking conditions, and using a routine protocol for tau analysis as described in Section 2.3.

Sensitivity of the Nano-iPCR for quantification of tau protein was tested by immobilizing recombinant tau at a concentration range from 1 to 100,000 pg/ml in TAU-5 antibody-coated wells of the PCR plate and then exposing the wells to Au-NPs functionalized with HT7 antibody and oligonucleotide primer 1. Detection by real-time PCR followed after washing off unbound particles. Fig. 1A shows a standard curve in the range of C_q values from 17.9 (at the tau concentration of 100,000 pg/ml) to 32.6 (at 1 pg/ml). Negative control (without tau protein) showed C_q values of about 33. The curve was linear from 10 to 10,000 pg/ml of recombinant tau protein with regression correlation coefficient (R^2) equal to 0.9918 (Fig. 1B). The LOD was found to be 5 pg/ml.

For comparison, recombinant tau protein was also quantified by ELISA-TSA using the same tau-specific antibody set (TAU-5 and HT7). Different concentrations of recombinant tau protein were added into wells with immobilized TAU-5. After incubation and washing off unbound tau, the wells were exposed sequentially to biotinylated anti-tau specific antibody HT7 and extravidin-peroxidase conjugates. The signal was enhanced by biotinyl-TSA. Absorbance values corresponding to different concentrations of tau protein (0–1200 pg/ml) are shown in Fig. 1C. The curve was linear in the range from 75 to 600 pg/ml of tau protein with regression correlation coefficient 0.9653 and LOD 140 pg/ml (Fig. 1D). The data clearly show that Nano-iPCR has a broader detection range and more than 30-fold higher sensitivity than ELISA-TSA. The broad detection range of Nano-iPCR was also demonstrated by precision profiles (Fig. 2E).

3.2. Quantification of tau protein in CSF by Nano-iPCR and comparison with ELISA

Next the amount of tau protein in 32 human CSF samples was examined by Nano-iPCR, ELISA-TSA and/or commercial ELISA. First we compared two ELISAs, ELISA-TSA and a commercial ELISA kit. Data presented in Fig. 2A show good correlation between tau concentrations as determined by ELISA-TSA (based on TAU-5 and HT7 antibodies) and the commercial kit with correlation coefficient $r = 0.636$ and $p < 0.0001$. This conclusion was corroborated by Bland–Altman plot (Fig. 2B). Precision, reproducibility and accuracy of ELISA-TSA was characterized by intra- and inter-assay CVs. The average of the intra-assay CV was 8.9% (range, 1.5-12%) for tau standards and 5.4% (range, 0–15%) for CSF samples. The average of inter-assay CV for ELISA-TSA based on CSF samples was 18% (range, 4–31%). Coefficient of variation between ELISA-TSA and ELISA kit based on CSF samples was 30.6% (range, 1–65%). The higher CV values could be caused by different standards used in the commercial ELISA kit and ELISA-TSA and different conditions for performing the assays.

Next, we compared the performance of Nano-iPCR and ELISA kit. Data presented in Fig. 2C show good correlation between tau concentrations as determined by Nano-iPCR and ELISA kit; correlation coefficient $r = 0.730$ and $p < 0.0001$. This finding was supported by the Bland–Altman plot (Fig. 2D). Precision, reproducibility and accuracy of Nano-iPCR were again characterized by intra- and inter-coefficients of variability. The average of intra-assay CV was 2.1% (range, 1–4%) for tau standards and 12.6% (range, 1–36%) for CSF samples. The inter-assay CV of Nano-iPCR based on CSF was 12.2% (range, 1.5–42%). Coefficient of variation between Nano-iPCR and ELISA kit was 23.4% (range, 2–63%). Again, the high CV

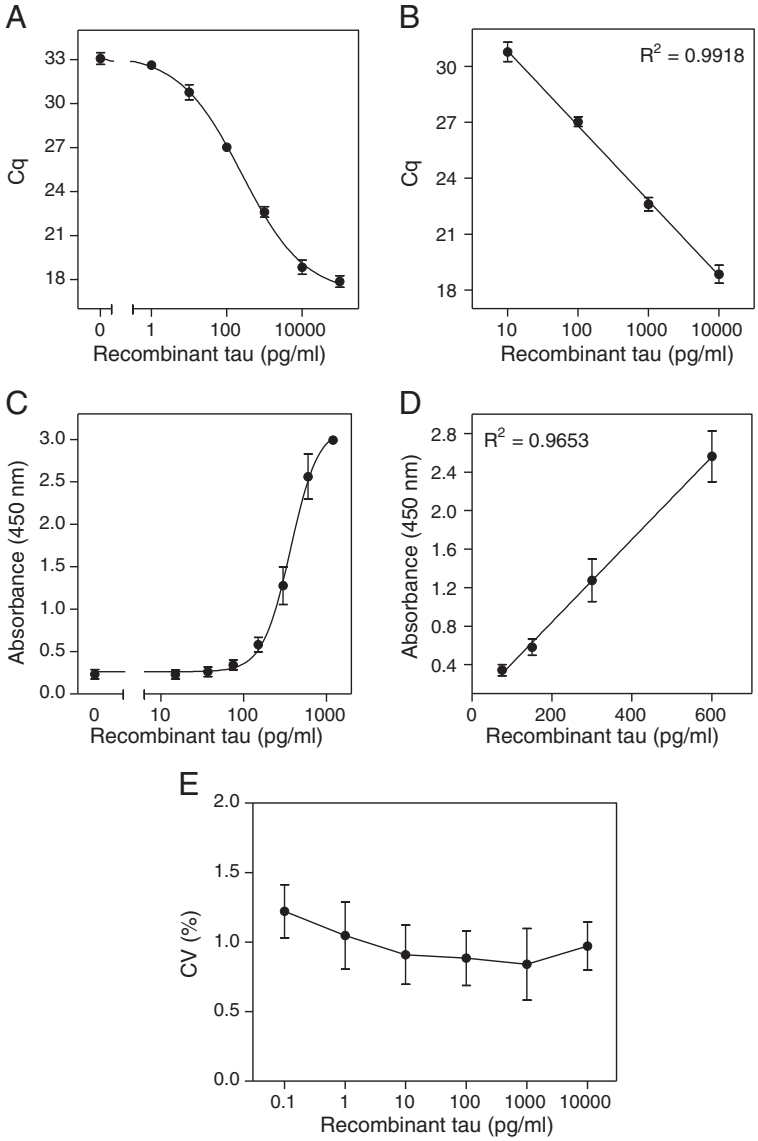

Fig. 1. Calibration curves of recombinant tau protein as determined by Nano-iPCR and ELISA-TSA. (A, B) Tau protein was detected by Nano-iPCR at concentrations ranging from 1 to 100,000 pg/ml (A); the curve was linear between 10 and 10,000 pg/ml (B). (C, D) Tau protein was detected by ELISA-TSA at a concentration range 15–1200 pg/ml (C); the curve was linear between 75 and 600 pg/ml (D). (E) Precision profile for Nano-iPCR. Estimated errors (expressed as % CVs) at various doses of recombinant tau protein were determined in each experiment from triplicates. Means ± SD were calculated from two independent experiments performed in triplicate (A, B) or duplicate (C, D), or six independent experiments (E).

between these two assays is probably caused by different standards and different assay conditions. For recovery experiments, high (4000 pg/ml), medium (800 pg/ml), and low (100 pg/ml) concentrations of recombinant tau protein were spiked into CSF samples. Tau levels were determined with Nano-iPCR with recovery (89%–113%). Finally, we attempted to determine the contribution of interfering factors present in CSF on linearity of the assay. We diluted CSF samples 2–16-fold and analyzed the amount of tau protein in the samples by Nano-iPCR. The observed mean value 107% (range, 91%–136%) indicated good linearity of the assay. These data indicate that Nano-iPCR gives results that could be used for determination of tau protein in clinical studies.

In conclusion, Nano-iPCR where Au-NPs functionalized with anti-tau monoclonal antibody and template oligonucleotide are used for real-time PCR detection of tau protein immobilized in wells of a PCR plate is fully suitable for tau protein detection. The assay is superior in sensitivity and detection range to ELISA-TSA based on the same set of anti-tau monoclonal antibodies or to a commercial ELISA kit. Nano-iPCR could be of importance for simplified determination of excessively high concentrations of tau protein in patients with Creutzfeldt–Jakob disease. The commercial availability of monoclonal antibodies suitable for tau protein detection by Nano-iPCR and the easy preparation of functionalized Au-NPs reduce the expenses for tau protein quantification at

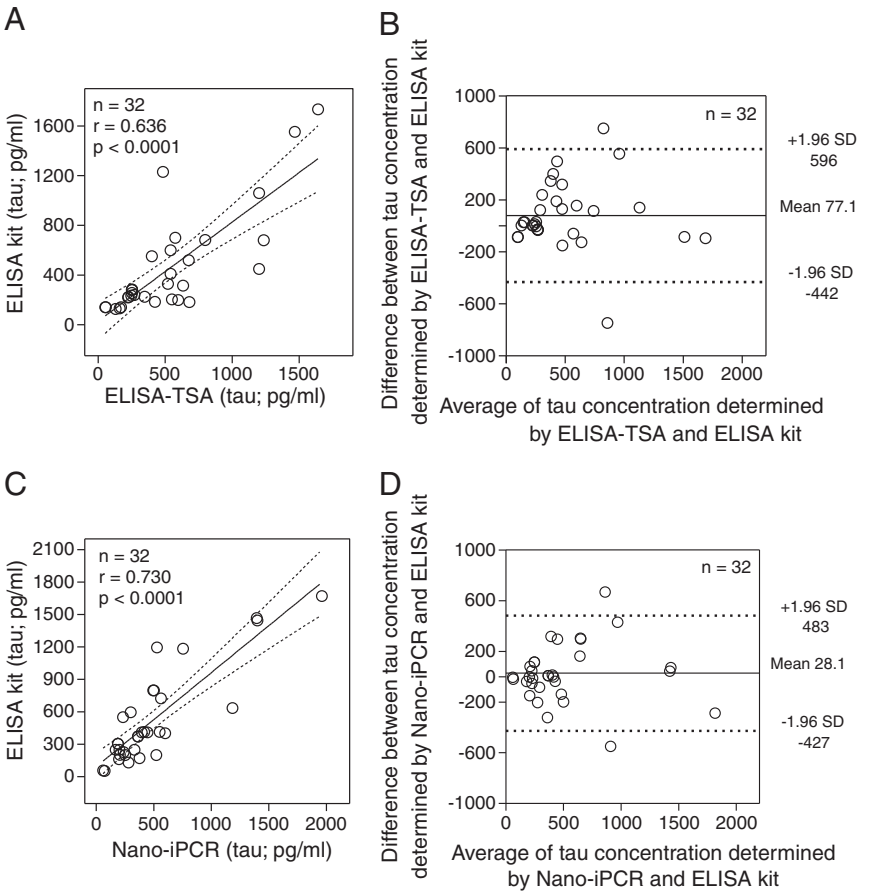

Fig. 2. Quantification of tau protein in CSF by various assays. (A, B) Comparison of ELISA-TSA and ELISA kit for tau protein level determination; (A) correlation plot and (B) Bland–Altman plot. (C, D) Comparison of Nano-iPCR and ELISA kit for tau protein level determination; correlation plot (C) and Bland–Altman plot (D). In correlation plots, the dashed line represents 95% line of identity; r means correlation coefficient; n means the number of samples analyzed. In Bland–Altman plots, the averages of the two methods of rating are shown along the horizontal axis and their difference along the vertical axis. The solid line represents the mean difference, and the upper and lower dotted lines represent 95% limits of agreement (±1.96 times the standard deviation of the differences).

least 10 times when compared to assays based on commercial kits.

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