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Feasibility of an early Alzheimer's disease immunosignature diagnostic test

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ABSTRACT

A practical diagnostic test is needed for early Alzheimer's disease (AD) detection. Immunosignaturing, a technology that employs antibody binding to a random-sequence peptide microarray, generates profiles that distinguish transgenic mice engineered with familial AD mutations (APPswe/PSEN1-dE9) from non-transgenic littermates. It can also detect an AD-like signature in humans. Here, we assess the changes in the immunosignature at different time points of the disease in mice and humans. We also evaluate the accuracy of the late-stage signature as a test to discriminate between young mice with familial AD mutations from non-transgenic littermates. Plasma samples from AD patients were assayed 3–12 months apart, while APPswe/PSEN1-dE9 and non-transgenic controls supplied plasma at monthly intervals until they reached 15 months of age. Microarrays with 10,000 random-sequence peptides were used to compare antibody binding patterns. These patterns gradually changed over the life-span of mice. Strong, characteristic signatures were observed in transgenic mice at early, mid and late stages, but these profiles had minimal overlap. The signature of young transgenic mice had an error rate of 18% at classifying plasma samples from late-stage transgenic mice. Conversely, the late-stage transgenic mice signature discriminated between young transgenic mice and littermates with an error rate of 21%. Less distinctive profiles were recognizable throughout the transgenic mice lifespan, being detectable as early as 2 months. The human signature had minimal change on short-term follow-up. Our results call for a reappraisal of the way incipient AD is studied, as biomarkers seen in late-stages of the disease may not be relevant in earlier stages.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia, affecting about 35.6 million people world-wide (Chui and Lee, 2002; Abbott, 2011). Because AD cannot be prevented or cured, the number of affected persons doubles every two decades, causing crippling cognitive disability and economic losses in excess of $604 billion per year. Early detection and treatment will be essential to control this problem (Buckholtz, 2011). In spite of recent advances (Shaw et al., 2007, 2009; Ewers et al., 2011), no specific tests are universally used to diagnose AD. As pathology slowly progresses for decades before initial symptoms emerge (Shaw et al., 2009), and since initial manifestations are generally subtle (Morris et al., 2001; Kawas, 2003; Grundman et al., 2004), a potential diagnostic test for AD must be highly sensitive. Given that future treatments are likely to target people with mild or no symptoms (Shaw et al., 2007, 2009; Buckholtz, 2011), the test must also be highly specific. Considering the challenges involved in obtaining samples from subjects with early AD stages, we explored the utility of a test developed using plasma samples from the terminal phase of the illness as a pre-symptomatic diagnostic tool.

Immunosignaturing is a general diagnostic technology which involves diluting blood and applying it to an array consisting of 10,000 random-sequence peptides (Legutki et al., 2010; Restrepo et al., 2011; Kukreja et al., 2012b; Stafford et al., 2012; Hughes et al., 2012). Antibodies bind to the array revealing a signature affected by the health status of the individual. The initial application of this technology showed that both transgenic mice with cerebral amyloidosis and humans with AD have distinctive immunosignatures relative to healthy age-matched controls (Restrepo et al., 2011), but no investigation of the signature stability over time was undertaken. Since the clinical diagnosis of AD is corroborated by autopsy in 65–80% of cases (Chui and Lee, 2002), a non-invasive blood test could be useful in clinical practice. More importantly, the application of this technology to the pre-symptomatic diagnosis of AD could help prevent or delay the onset of dementia if disease-modifying therapies become available. The simplest approach to developing such a test is to use the signature of autopsy-confirmed AD to create an indicator for early stages of dementia. Here we use a mouse model of AD, APPswe/PSEN1-dE9 mice, to explore this possibility.

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2. Materials and methods

2.1. Microarray

The protocols, performance, sample preparation methods and statistical analyses of the technology are described elsewhere (Brown et al., 2011; Restrepo et al., 2011; Halperin et al., 2012; Hughes et al., 2012; Kroening et al., 2012; Kukreja et al., 2012a,b; Stafford et al., 2012). Briefly, an immunoassay was developed using 10,000 random-sequence 20-mers covalently attached to a glass slide. Peptides were designed with random sequences, except for glycine–serine–cysteine linkers at the carboxyl (peptide library 1) or amino (library 2) terminus. Library 1 peptides were synthesized by Alta Biosciences (Birmingham, UK), spotted in duplicate using a NanoPrint LM60 microarray printer (Arrayit, Sunnyvale, CA). Library 2 peptides were synthesized by Sigma Genosys (St. Louis, MO), printed by Applied Microarrays (Tempe, AZ) using a piezo non-contact printer in a two-up design. Slides were pre-washed with 33% isopropanol/7.5% acetonitrile/0.5% trifluoroacetic acid, and blocked with 0.015% mercaptohexanol/3% BSA/0.05% Tween 20 in PBS prior to adding plasma at 1:500 dilution in 3% BSA/0.05% Tween 20 PBS. Experiments were carried out in a TECAN HS4800-Pro automated incubator (Tecan, Männedorf, Switzerland). Biotinylated anti-human (Bethyl, Montgomery, TX) or anti-mouse (KPL, Gaithersburg, MD) antibodies were incubated with slides, washed, then followed by incubation with streptavidin-Alexa-647 (Invitrogen, Carlsbad, CA). Slides were scanned with an Agilent 'C' scanner (Agilent Technologies, Santa Clara, CA), generating digital images that were subsequently processed with GenePixPro6.0 (Molecular Devices, Palo Alto, CA) and analyzed in GeneSpring 7.3.1 (Agilent, Santa Clara, CA).

2.2. Analysis

Once data was imported into GeneSpring, signal intensity was $\log_{10}$-transformed and median-normalized before analysis. Pearson's correlation coefficient was calculated across replicate slides to ensure reproducibility ≥ 0.85. Technical replicates < 0.85 were reprocessed. Multivariate clustering (two-way hierarchical clustering) was used to generate heatmaps using Euclidean distance with complete linkage as the measure of similarity, while principal component analysis (PCA) was used to display relative differences across samples. Figs. 1–3 utilize plots of the first three principal components. Peptide microarrays provided <1.3-fold minimum average detectable fold change at $\alpha = 0.05$ and $\beta = 0.20$ per 2 technical replicates. For classification we used linear discriminant analysis (LDA) in R with leave-one-out cross validation to estimate error. *T*-test with FWER (family-wise error rate) of 5% is used to correct for false positives due to multiple testing. The *p*-values presented in this paper are not raw but have been adjusted by the Benjamini and Hochberg false discovery rate algorithm (Benjamini and Hochberg, 1995).

2.3. Human plasma

Plasma samples from 6 AD patients and 5 age-matched controls without cognitive derangement enrolled in a brain-bank program were provided by Alex Roher (Banner Sun Health Research Institute, Phoenix, AZ). Postmortem examination was performed by a neuropathologist on 9 patients. Samples were acquired after written consent and approval from the Banner Institutional Review Board (IRB). Profiling studies were approved by ASU's IRB (protocol# 0912004625).

2.4. Mice

Female APPswe/PSEN1-1dE9 TGM and B6C3F1/J non-transgenic controls (n = 5/group) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed with standard chow and water provided ad libitum. Plasma samples were processed from blood obtained via submandibular puncture at monthly intervals beginning at age 2 months and stored at −80 °C. Mice were sacrificed at 15 months of age through intra-peritoneal injection of tribromoethanol (5 mg) followed by intra-cardiac exsanguination and cold PBS perfusion prior to decapitation for brain harvesting. Brain axial sections (3–4 mm thick) were treated with 10% formaldehyde overnight, followed by paraffin processing for immunostaining. Murine experiments were conducted under a protocol approved by the Arizona State University Institutional Animal Care and Use Committee.

3. Results

Results from immunosignaturing assays require an understanding of the characteristics of the technology. While expression or SNP microarrays demonstrate a one-to-one binding between RNA or DNA and the target probe, the immunosignaturing peptide arrays enable multiple specificities of antibody to bind a single peptide while a single antibody may bind multiple peptides (Kukreja et al., 2012b). This effect is accommodated by the statistical methods used to select peptides and is noted as the "Immunosignaturing Effect" (Stafford et al., 2012).

3.1. Stability of human immunosignature

We first asked whether the AD immunosignature in humans is stable over time. To answer this question, we assayed two plasma samples collected between 3 and 12 months apart from 5 patients with AD (4 autopsy-confirmed), 6 normal elderly controls (4 autopsy-confirmed), and a demented patient with signs of progressive supranuclear palsy (PSP) on autopsy. The time 0 samples were used in a previous paper which examined whether a signature of AD existed at all (Restrepo et al., 2011). Fifty peptides were selected by a two-tailed *T*-test with FWER = 5% ($p < 4.28 \times 10^{-10}$) patients from age-matched controls using both time 0 and follow-up samples. LDA using these peptides yielded a 0% misclassification rate. We also tested for differences between time 0 and follow-up using a *T*-test between 'early' and 'late' time points. No peptides passed multiple testing criteria. Fig. 1 (left panel) shows the resulting heatmap where hierarchical clustering was done using Euclidean distance as the measure of similarity. Clustering was performed on the peptides (vertical axis) and patients (horizontal axis) with the colored bars representing the patient class. Patients showed a strong tendency to group with their follow-up sample. A PCA demonstrates this effect in Fig. 1 (right panel). A secondary effect, which is a trait of the immunosignaturing technology, is the higher dispersion of points in healthy cohorts, and the relatively tighter grouping of patients in the disease cohorts (Stafford et al., 2012). This demonstrates that the immunosignature of Alzheimer's disease is stable over at least the time course of this collection study. This has not been shown for any other ongoing or completed immunosignaturing study of chronic disease. The PSP patient exhibited an intermediate pattern, although peptides were selected for resolving AD, not PSP. Eight out of these fifty peptides also bind antibodies raised against Aβ, suggesting that part of the signature could involve anti-Aβ immune-reactivity (data not shown and Restrepo et al., 2011). These observations suggest that AD plasma contains an immunosignature that can distinguish AD people from non-AD controls reproducibly over time.

3.2. Time course of immunosignatures in APPswe/PSEN1-dE9 mice

Because human plasma samples spanning the entire AD's time course are rare and difficult to obtain, we used APPswe/PSEN1-dE9 transgenic mice, a well-characterized animal model of AD engineered with two human mutations leading to accelerated cerebral amyloidosis (Jankowsky et al., 2004; Qu et al., 2004, 2006, 2007; Reiserer et al., 2007; Gotz and Ittner, 2008) to ask two related questions relevant to