

Identification of novel candidate autoantibodies in Alzheimer's disease

B. Z. Wang^a , F. Z. Zailan^a , B. Y. X. Wong^a , K. P. Ng^a and N. Kandiah^{a,b,c} 

^aDepartment of Neurology, National Neuroscience Institute, Singapore; ^bDuke-NUS Graduate Medical School, Singapore; and ^cNTU-Imperial Lee Kong Chian School of Medicine, Singapore, Singapore

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Background and purpose: Accumulated failures in Alzheimer's disease (AD) clinical trials have highlighted an urgent need to identify additional biomarkers involved in AD. Recently, mounting evidence reported that autoantibodies are ubiquitous in human sera. However, it is unknown whether autoantibodies are upregulated in amyloid-tau biomarker-confirmed AD.

Methods: A total of 40 subjects with mild dementia (Clinical Dementia Rating = 1) were stratified into AD ($n = 16$) and non-AD ($n = 24$) groups according to their cerebrospinal fluid levels of tau and A β_{42} . Their sera were collected and analyzed using a microarray containing > 1600 potential human autoantigens. Autoantibodies that were present exclusively in the AD group were identified and selected using the penetrance-based fold change method with the following criteria: penetrance fold change_(AD) ≥ 2 , frequency_(AD) $\geq 15\%$ and frequency_(non-AD) = 0%.

Results: All controls and samples passed the quality control criteria and were further used for biomarker analysis. Six autoantibodies with elevated responses to the following autoantigens were found exclusively in the AD group: nucleosome assembly protein 1-like 3 (31.3%, 5/16 subjects) and microtubule-associated protein 4, pantothenic acid kinase 3, phosphoinositide-3-kinase regulatory subunit 1, protein tyrosine phosphatase type IVA member 1 and SRY (sex-determining region Y)-box 15 (all 18.8%, 3/16 subjects).

Conclusions: Although some identified autoantigens are linked to AD and cognitive dysfunction, the increased autoantibody levels have not been reported in AD. Autoantibodies may provide deeper insights into the pathogenesis of AD and serve as diagnostic biomarkers; their corresponding antigens can be further studied to assess their potential as therapeutic targets.

Introduction

Currently, monotherapies targeting A β_{42} or tau alone have been unsuccessful in clinical trials [1], suggesting that Alzheimer's disease (AD) is a complex disease involving multiple proteins and processes. Therefore, there is an urgent need to identify additional biomarkers involved in AD.

Recent evidence reported that autoantibodies are ubiquitous in human sera regardless of age, sex and

disease status [2]. Indeed, autoantibodies can discriminate between patients with AD and non-demented controls [3] as well as predict the risk of progression from mild cognitive impairment to AD [4,5]. Their utility in diagnosing AD throughout the entire disease course has also been reported [6]. However, previous studies with autoantibodies were based on clinical diagnosis of AD and not on biomarker-based diagnosis [3–6]. The use of traditional cerebrospinal fluid (CSF) biomarkers such as tau and A β_{42} will allow for a specific diagnosis of AD and thus help to identify autoantibodies with a more definite role in AD pathogenesis. In this pilot study, we sought to examine whether autoantibodies

Correspondence: N. Kandiah, Department of Neurology, National Neuroscience Institute, 11 Jalan Tan Tock Seng, 308433, Singapore (tel.: +65 6357 7171; fax: +65 6357 7137; e-mail: nagaendran.kandiah@singhealth.com.sg).

were upregulated in an amyloid-tau biomarker-confirmed AD cohort.

Materials and methods

Study population

The study complied with the tenets of the Declaration of Helsinki and was approved by the Centralized Institutional Review Board (CIRB) (#2015/2218). All subjects provided written informed consent.

Subjects with mild dementia (Clinical Dementia Rating = 1) were recruited from a tertiary neurology center in Singapore. Inclusion criteria were symptom onset occurring before 65 years of age with a reliable caregiver to provide collaborative history. Subjects with any major systemic or psychiatric disease were excluded. Subjects were classified into AD ($n = 16$) and non-AD ($n = 24$) groups using Duits ratio (total-tau:A $\beta_{42} > 0.52$), which has been shown to robustly differentiate between those with and without AD dementia using CSF amyloid and tau [7].

Cerebrospinal fluid and blood processing

Upon enrollment, blood was collected by a trained phlebotomist and CSF was collected via lumbar puncture. Blood tubes were left to stand at room temperature (25°C) for 30 min. Blood and CSF samples were centrifuged at 2000 g for 10 min at 4°C and the resulting serum and supernatant were aliquoted and stored at -80°C. CSF levels of t-tau, p-tau_(T181) and A β_{42} were measured using INNOTEST® (Fujirebio Europe N.V., Zwijnaarde, Belgium) according to the manufacturer's instructions [8].

Autoantibody microarray

A functional protein microarray platform (Immuno-me™) was developed using Sengenics patented KREX™ technology [9,10], which has >1600 human autoantigens making it suitable for autoantibody profiling [11,12]. Hybridization signals were measured with a microarray laser scanner (Agilent Technologies, Santa Clara, CA, USA) at 10- μ m resolution. Fluorescence levels were detected according to the manufacturer's instructions using the Feature Extraction software (Agilent Technologies).

Selection of autoantibody biomarkers

Biomarker selection was based on the penetrance-based fold change analysis method, which sets a protein-specific threshold (i.e. background threshold) using healthy control samples. Autoantibodies found exclusively in

the AD group were identified and selected using the penetrance-based fold change method with the following criteria: penetrance fold change_(AD) ≥ 2 , frequency_(AD) $\geq 15\%$, frequency_(non-AD) = 0%.

Statistical analysis

Statistical analyses were performed with SPSS (v.25, Armonk, NY, USA). Proportions and means were computed for categorical and continuous variables, respectively. Group differences were analyzed using the Mann-Whitney test. The Chi-squared test was performed for categorical data. All P -values were two-tailed with $P < 0.05$ considered statistically significant.

Results

Demographic and clinical characteristics

A total of 40 subjects were included and classified into two groups, i.e. AD ($n = 16$) and non-AD ($n = 24$) dementia. Global cognitive scores and ApoE presence did not differ between the groups (Table 1, all $P > 0.05$). All patients with AD had Duits total-tau:A β_{42} ratio > 0.52 .

Elevated autoantibody responses exclusive to the Alzheimer's disease dementia group

We found increased autoantibody responses to six autoantigens in the AD group (Fig. 1a). The identified

Table 1 Demographics and clinical characteristics

	<i>n</i>	Non-AD dementia	<i>n</i>	AD dementia	<i>P</i> value
Age (years)	24	57.1 \pm 1.5	16	55.1 \pm 1.4	0.282
Sex, male	24	8 (62.5%)	16	15 (50.0%)	0.433
Education (years)	22	13.7 \pm 0.9	16	14.1 \pm 1.3	0.858
Cognitive scores (max.)					
MMSE (30)	21	25.1 \pm 0.8	15	26.6 \pm 0.9	0.140
MoCA _{adj} (30)	21	23.4 \pm 1.0	15	23.8 \pm 1.3	0.872
CSF biomarkers (pg/mL)					
A β_{42}	24	847.3 \pm 39.0	16	649.9 \pm 95.5	<0.001
Total-tau	24	236.7 \pm 16.6	16	704.1 \pm 118.2	<0.001
Phospho-tau _(T181)	24	38.2 \pm 3.1	16	87.1 \pm 12.5	<0.001
Duits total-tau:A β_{42} ratio	24	0.3 \pm 0.0	16	1.4 \pm 0.3	<0.001
ApoE presence					
$\epsilon 2$	24	3 (12.5%)	14	2 (14.3%)	0.875
$\epsilon 3$	24	23 (95.8%)	14	14 (100.0%)	0.439
$\epsilon 4$	24	5 (20.8%)	14	5 (35.7%)	0.315

Data are given as mean \pm SEM and n (%), all P two-tailed. Categorical variables were analyzed using the chi-squared test, whereas continuous variables were analyzed using the Mann-Whitney U -test. AD, Alzheimer's disease; CSF, cerebrospinal fluid; MMSE, mini mental state exam; MoCA_{adj}, Montreal cognitive assessment adjusted for age.

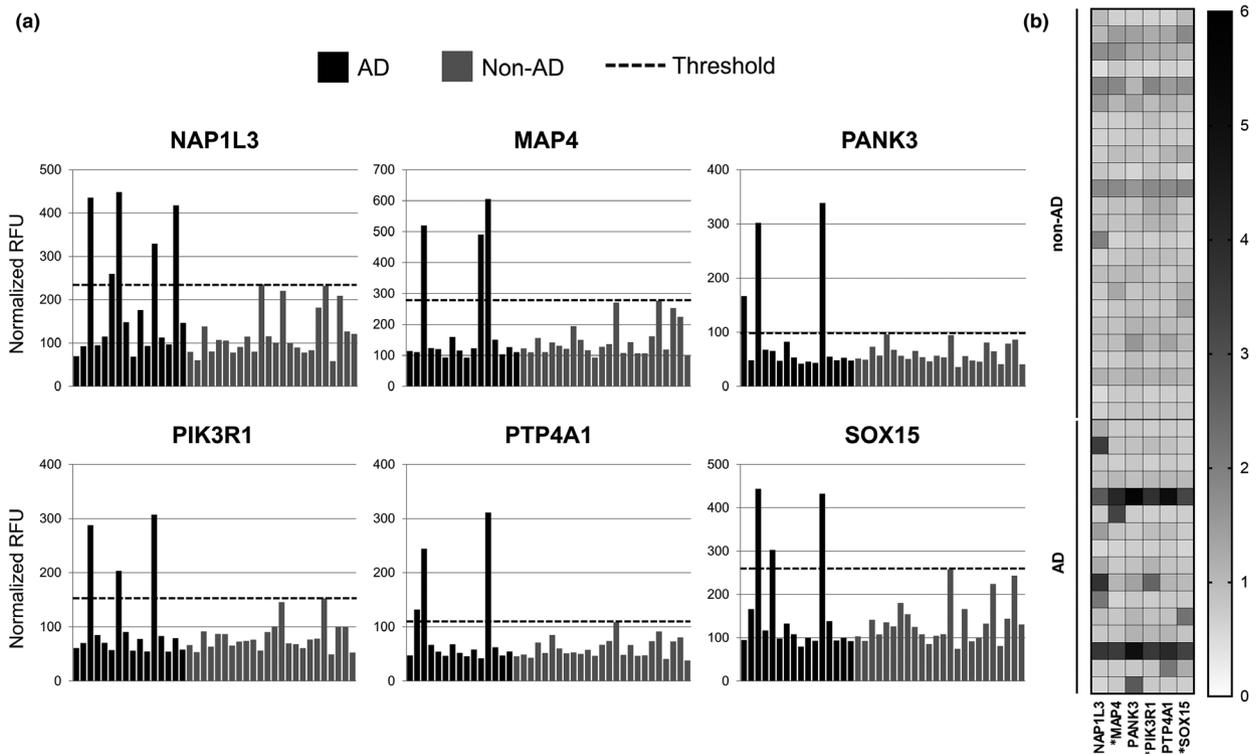


Figure 1 Autoantibodies identified exclusively in the Alzheimer's disease (AD) dementia group. (a) One bar represents one subject. Black bars indicate AD dementia group; grey bars indicate non-AD dementia group. Dashed line shows threshold set at +2 SD of non-AD samples. Any bar crossing the threshold met the selection criteria using the penetrance-based fold change (pFC) method. (b) Heatmap of the six identified autoantigens in the AD dementia group. Increasing color intensity indicates higher fold change in subjects with AD vs. subjects without AD. *Linked to AD and cognitive dysfunction, but autoantibody responses toward them have not been reported. MAP4, microtubule-associated protein 4; NAP1L3, nucleosome assembly protein 1-like 3; PANK3, pantothenic acid kinase 3; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTP4A1, protein tyrosine phosphatase type IVA member 1; RFU, relative fluorescence units; SOX15, SRY (sex-determining region Y)-box 15.

autoantigens were found to be involved in different biological processes (Fig. 1b) including transcriptional regulation [SRY (sex-determining region Y)-box 15 (SOX15)], metabolism [pantothenic acid kinase 3 (PANK3)], cytoskeleton organization [microtubule-associated protein 4 (MAP4)], cell cycle [nucleosome assembly protein 1-like 3 (NAP1L3)] and cell signaling [protein tyrosine phosphatase type IVA member 1 (PTP4A1) and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1)]. SOX15, MAP4 and PIK3R1 have been linked to AD and cognitive dysfunction using the Open Targets Platform [13], but autoantibody responses toward them have not been reported.

Discussion

The findings from this pilot study demonstrated that six novel serum autoantibodies were upregulated exclusively in AD dementia, suggesting that elevated autoantibody responses can be used as biomarkers for AD diagnosis. However, not all patients with AD had

autoantibodies reactive to these six antigens, reiterating the complexity of processes and proteins involved in AD pathogenesis.

We previously showed that the association of *APOE* $\epsilon 4$ with the high-mobility group box 1 (HMGB1) protein led to widespread cortical thinning in patients with mild cognitive impairment [14]. It is plausible that SOX15, belonging to a family of transcription factors [15], can act as a transcriptional regulator of HMGB1. Dysregulation of SOX15 could lead to dysfunctional HMGB1 transcriptional activity and thus cell death resulting in cortical thinning. PTP4A1 has been implicated in synaptic transmission and plasticity. Decreased gene expressions of *Ptp4a1* along with other cAMP response element-binding protein (CREB)-dependent genes were seen in the hippocampus of trained *APP_{Sw}*, *Ind* mice, which were associated with early memory loss [16]. Further evidence demonstrated that PTP4A1 (also known as phosphatase of regenerating liver-1) was important for synapse formation via modulation of insulin receptor-Akt signaling in *Drosophila*; loss of

phosphatase of regenerating liver-1 resulted in locomotor deficiencies and decreased numbers of synapses [17]. Therefore, dysregulation of PTP4A1 may directly cause the loss of synapses leading to the memory loss characteristic of AD dementia. Genome-wide association studies have highlighted the involvement of PIK3R1 in A β production [18] and deposition possibly through the disruption of the insulin signaling pathway [19]. Moreover, there was a significant association between the homozygous polymorphic variation in PIK3R1 (Met326Tyr) and the genetic risk for late-onset AD [20]. Due to the role of PIK3R1 in the insulin signaling pathway, these suggest that PIK3R1 is a key player in the exacerbation of AD pathology and potentially more so in *APOE* ϵ 4 carriers with type 2 diabetes [21]. PANK3 catalyzes the first committed step in the synthesis of coenzyme A, which is essential for a variety of metabolic processes including fatty acid synthesis and oxidation [22]. A study examining the metabolome of AD brains revealed that the pantothenate and coenzyme A biosynthesis pathway was one of the most affected pathways in the frontal cortex of subjects with AD vs. cognitively normal (CN) subjects [23]. Compared with CN subjects, AD subjects had a substantially higher level of pantothenate, which could be attributed to PANK3 dysfunction. The exact role of PANK3 in AD dementia remains to be elucidated; we hypothesize that a defect in PANK3 could lead to the accumulation of intracellular pantothenate and decreased synthesis of coenzyme A, resulting in the dysregulation of downstream metabolic pathways and, ultimately, cell death. RNA-seq analysis showed high expression of MAP4 in the brains of healthy individuals, second only to the heart [24]. MAP4 expression is low during developmental stages but becomes high in the adult brain and can be found in neurons and glia [25]. Using the Open Targets Platform with MAP4 as the search term, gene expression analysis of MAP4 was found to decrease by 1.5-fold in the hippocampus and mid-temporal gyrus but increased by 2-fold in the superior frontal gyrus in subjects with AD compared with normal subjects, indicating that regional gene expression of MAP4 plays a role in AD pathology. NAP1L3 belongs to a family of nucleosome assembly proteins [26] and has not been studied as extensively as NAP1L1. Using GeneCards [27] with NAP1L3 as the search term, RNA-seq analysis showed that it is highly expressed in the brain especially in the amygdala and prefrontal cortex. In addition, a study reported that a decrease in NAP1L3 gene expression in human umbilical cord hematopoietic stem cells revealed the induction of gene expression signatures associated with cell cycle progression [28]. Taken together, we propose that a dysfunction in NAP1L3, similar to its relative

NAP1L1, can lead to a dysregulation of cell cycle proteins such as Cdk5 to phosphorylate tau [29] and promote tau aggregation [30] in the amygdala, potentially causing depressive symptoms and influencing cognitive outcomes in patients with mild cognitive impairment, which we have shown previously [31].

Our study has one main limitation. This was a relatively small study that lacked CN subjects, which limits the generalizability of our results. We intended this to be a pilot study to address the question of whether autoantibodies can be potential biomarkers for an amyloid-tau biomarker-confirmed AD cohort. A larger study including CN subjects will be needed to ascertain the usefulness of the biomarkers that we identified here.

Autoantibody research is important and newer approaches such as autoantibodies against neurotransmitters and lipids [32] can be innovative. Future studies should also consider age, sex, ethnicity, disease severity and presence of co-morbidities as factors that can influence autoantibody levels [2,33,34]. Thus, care should be taken to generalize our findings as our AD group is young (mean age 55 years) and could be a source of discrepancy between this study and others.

In conclusion, we identified, for the first time, novel autoantibody biomarkers in an amyloid-tau-confirmed AD cohort that may serve as diagnostic biomarkers and recommend further study to determine the potential of the corresponding autoantigens as therapeutic targets.

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Disclosure of conflicts of interest

B.Z.W., F.Z.Z., B.Y.X.W. and K.P.N. declare no financial or other conflicts of interest. N.K. reports grants, personal fees and non-financial support from Novartis Pharmaceuticals, Schwabe Pharmaceuticals, Eisai Pharmaceuticals and Lundbeck Pharmaceuticals, as well as personal fees from General Electric, outside the submitted work.

Data sharing

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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