

Individual PKR Activation in Peripheral Blood Mononuclear Cells Correlates with Cognitive Decline in Alzheimer's Disease

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Abstract

Background: Among the pathogenic processes in Alzheimer's Disease (AD), systemic inflammation, autophagy and metabolic alterations have been described. The double-stranded RNA-dependent protein kinase (PKR) is an ubiquitous cellular kinase involved in these several pathogenic processes, and previous studies reported its involvement in AD. PKR can be activated by phosphorylation (P_{T451}-PKR) and cleaved in catalytic form (PKR-KD). We have previously described a correlation between PKR activation in peripheral blood mononuclear cells (PBMCs) and cognitive status in advanced AD. However, the link between PKR's activation in PBMCs and cognitive decline in AD remains unknown.

Methods: Thirty-one patients with AD were included. Acute and chronic inflammations were considered as exclusion criteria. Cognitive status was assessed with Mini Mental State Examination (MMSE) and AD assessment scale (ADAScog) at baseline, 6, 12 and 24 months of follow-up. PKR expression and its activation were measured using western blot at baseline, 12 and 24 months.

Results: At baseline, MMSE mean score was 20.5 ± 2.6; Except for total PKR expression between baseline and M12, no longitudinal significant variation of PKR activation was found. Higher PKR-KD activation was correlated with faster cognitive decline at an individual level at M6 (r=0.33, p=0.0453), M12 (r=0.48, p=0.0033) and M24 (r=0.36, p=0.0407). Other PKR forms were correlated with cognitive decline at M12.

Conclusions: Our results suggest that PKR activation in PBMCs can predict cognitive decline in AD at an early stage at an individual level.

Keywords: Alzheimer's disease; Cognitive decline; Chronic inflammations; Cognitive disorders

Introduction

Alzheimer's disease (AD) is a neurodegenerative pathology marked by cognitive disorders. *Post mortem* studies of AD patients show senile plaques and neurofibrillary tangles. Cause of neuronal degeneration is not completely resolved and many pathogenic mechanisms like inflammation [1], autophagy [2] or metabolic alterations [3] are involved.

The double-stranded RNA-dependent protein kinase (PKR) is an ubiquitous cellular kinase involved in several pathways like inflammation [4], apoptosis [5] and protein synthesis [6]. PKR can be activated by Protein Activator of the Interferon-Induced Protein Kinase (PACT) in a phosphorylated form (P_{T451}-PKR) [7], full length P_{T451}-PKR can be cleaved in a more active form (C terminal catalytic kinase domain (KD), P_{T451}-PKR-KD) [8]. PKR is involved in inflammation *via* nuclear factor-kappa B (NF-κB) pathway activation through interaction with the Inhibitor of KappaB Kinase beta (IKKβ), a subunit of the IKK complex, leading to nuclear translocation of NF-κB [4]. PKR activation down-regulates translation by eIF2α phosphorylation [6]. In Peripheral Blood Mononuclear Cells (PBMCs) from AD patients, in tri-culture of neurons/astrocytes/microglia and in transgenic mice, treatment with a specific PKR inhibitor highly decreases pro-inflammatory cytokine production [9-11]. *In vitro* studies showed relationship between PKR and AD specific lesions. In cell culture, Aβ exposition induces PKR activation which leads to apoptosis [12]. In return, PKR seems involved in Aβ production by the regulation of BACE1 [13]. Similarly, PKR after activation by Glycogen synthase kinase 3 beta (GSK-3β) participates in Tau phosphorylation [14].

Several clinical studies suggest an involvement of PKR in the physiopathology of AD. *Post mortem* studies show a raise of phosphorylated PKR co-localizing with phosphorylated [15]. In Cerebrospinal Fluid (CSF), levels of PKR and P-PKR discriminate control from AD patients [16]. Furthermore in longitudinal study, high CSF phosphorylated PKR levels were associated with faster cognitive decline [17]. CSF is not routinely collected in clinical practice and blood sample is less invasive and easier to perform. Our previous results showed PKR involvement in PBMCs of AD patients with a negative correlation between the level of PKR activation and cognitive test scores [18]. The aim of this study was to determine relationship between PKR and its activation in PBMCs with the cognitive decline through a 2-years follow-up of AD patients.

Materials and Methods

Chemical

Ficoll Histopaque[®] 1077, newborn calf serum heat inactivated,

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sodium fluoride (NaF), phenylmethylsulfonyl fluoride (PMSF), protease and phosphatase inhibitor cocktails, sterile filtered dimethylsulfoxide HybriMax[®] (DMSO), Triton X100, dithiothreitol (DTT) and all reagent grade chemicals for buffers were obtained from Sigma (St Quentin Fallavier, France); RPMI 1640 medium, L-glutamine, 5000 units of penicillin (base) and 5000µg of streptomycin (base)/mL mixture, Quant-it protein assay, NuPAGE 4-12% Bis Tris 1.5 mm gel, NuPAGE antioxidant, MES SDS running buffer, NuPAGE iBlot[™] nitrocellulose Transfer Stacks from Gibco-Invitrogen (Fisher Bioblock Scientific distributor, Illkirch, France). For western blot, primary antibodies rabbit anti-P_{T451}PKR as custom antibodies produced by Eurogentec (Seraing - BELGIUM), rabbit anti-PKR from Cell Signalling (Ozyme, St Quentin Yvelines, France) and mouse anti-β-actine from Sigma (St Quentin Fallavier, France). Secondary antirabbit IgG antibody conjugated with Horseradish Peroxydase were purchased from Cell Signalling (Ozyme, St Quentin Yvelines, France), secondary anti-mouse IgG conjugated with Horseradish Peroxydase and ECL plus kits were purchased from GE Healthcare Europe GmbH (Velizy Villacoublay, France).

Patients

Patients were selected between November 2010 and December 2012 in the memory center of Poitiers University Hospital in an ancillary study of a national clinical research project (CYTOCOGMA). AD was diagnosed according to NINCD-RDS-ADRDA criteria with a MMSE score between 16 and 25 at inclusion. Exclusion criteria were any other neurological disease than AD, ongoing inflammatory state or anti-inflammatory treatment.

Patients had a clinical and neuropsychological assessment with MMSE and Alzheimer's disease assessment scale (ADAScog) (the higher the score the lower the performance) at diagnosis, after 6 months, one year and two years of follow-up. Biological assessment was conducted at baseline and after one and two years.

PBMCs extraction, culture and cellular lysis

Methods have been extensively used in our laboratory and previously described [9,19-21]. PBMCs were isolated by Ficoll Histopaque[®] density gradient centrifugation within two hours after blood test. Using a KOVA cell, cells were counted and seeded at 10⁶ cells/well in six-well plates. After 48 hours of culture, cells were isolated from the medium by centrifugation. Cells were lysed using a lysis buffer (50 mM Trizma[®] base, 50mM NaCl pH 6.8, 1% Triton X100, 1mM PMSF, 50mM NaF, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail). Proteins levels were assessed using Quant-it[®] protein assay. Samples were preserved in the freezer 80 degrees.

Western blot

Each sample was taken for a quantity of 30 µg of proteins per well and diluted with electrophoresis buffer containing 0.05M DTT. Each sample was boiled at 100°C during 5 minutes. Proteins were separated on NuPAGE gel with 200 V during 35 minutes. Proteins were transferred to NuPAGE iBlot[™] nitrocellulose Transfer Stacks using iBlot[™] Gel Transfer Device. Membranes were blocked for two hours at Room Temperature (RT) in Tris-buffered saline/Tween (TBST: 20mM Tris-HCl, 150mM

NaCl, pH 7.5, 0.05% Tween 20) containing 5% BSA and 0.21% NaF. Membranes were incubated with primary antibodies in blocking buffer overnight on a shaker at 4°C. The primary antibodies used were rabbit anti-P_{T451}PKR (1:100) which detect threonine 451 phosphorylated from both full-length (74 kDa) and catalytic fragment of PKR (35 kDa). It was demonstrated that the released KD domain of PKR is constitutively active [22], which is well supported by structure studies [23]. Moreover, the caspase-generated fragments of PKR cooperate to activate full-length PKR and amplify the translation inhibitory signal [8]. Other primary antibodies rabbit anti-total PKR (1:500), mouse anti-β-actine (1:100,000) were also used. Membranes were washed twice with TBST during 10 minutes at RT on a shaker. Membranes were incubated with secondary antibodies conjugated with the peroxidase according to the primary antibody origin during one hour at RT. The second antibodies used were goat anti-rabbit IgG (1:1000) and sheep anti-mouse IgG (1:1000). Membranes were washed twice and incubated with chemiluminescence ECL plus[®] system, luminescent signal was captured by Gene Snap software and analyzed by Gene Tools software, both with Gbox system[®] (Syngene, Ozyme distributor, France). Protein expression level was reported to the actin expression level and expressed in arbitrary units. Activation of full length PKR was calculated using the ratio between P_{T451}-PKR and total PKR. Activation of PKR-KD was calculated using the ratio between P_{T451}-PKR-KD and total PKR.

Statistical analysis

All statistical analyses were carried out using the SAS 9.2 software package (SAS Inc., Cary, NC, USA). Quantitative variables are described with mean and Standard Deviation (SD). Biological values measured at baseline, M12 and M24 for AD patients were compared by non-parametric Friedman's test for repeated measures followed by post-hoc Sheffé's test if necessary. The correlations between the expression of total PKR or its phosphorylated forms, its activations (full-length PKR and PKR-KD) and cognitive scores were investigated with the Spearman correlation coefficient (rho) calculation. The level of significance was p<0.05.

Results

Patients characteristics

Thirty-seven patients were included, among which thirty-one were followed during two years. Two patients died and 4 were lost to follow-up. Mean age at baseline (D0) was 78.3 years. Table 1 shows neuropsychological characteristics at D0, after 6 months (M6), and one and two years of follow-up (M12 and M24). During the two years follow-up we observed an average decrease of 3.7 points for MMSE and an average increase of 4.2 points for ADAScog.

Longitudinal PKR activation in PBMCs of AD patients

PKR consists of two functionally distinct domains: An aminoterminal regulatory domain and a carboxyl-terminal catalytic domain [24]. The regulatory domain consists of two dsRNA-binding motifs, followed by a spacer. Binding of dsRNA exposes the catalytic site and induces dimerization of PKR. Dimerization allows autophosphorylation, rendering PKR active [25]. However, dsRNA-independent protein activators of PKR, such as PACT have been identified [7]. The T451 site

Mean (SD) [Extremes]	D0	M6	M12	M24
	n = 31	n =28	n =31	n = 29
MMSE	20.5 (2.6) [16-25]	19.7 (3.5) [13-25]	19.1 (3.9) [12-26]	16.8 (4.9) [7-25]
ADAScog	15.6 (5.9) [6.9-28.5]	16.4 (7.3) [7.3-36.8]	16.7 (7) [5.9-31]	19.8 (10.1) [6-42.9]

Table 1: Clinical and neuropsychological characteristics of patients.

of PKR is one of the first active sites leading to its autophosphorylation at different sites and homodimer formation, resulting in its activation [26]. Figure 1 shows PKR expression and its activation at baseline and during the follow-up. A Friedman's test indicated significant differences for expression of total PKR ($p=0.0001$), full-length P_{T451} PKR ($p=0.0247$) and P_{T451} -PKR-KD activation ($p=0.0053$) during the follow-up. However, a Scheffé's post-hoc test revealed that only the levels of total PKR expression at D0 was significant to those at M12 (increase of 215% at M12). The decrease of the P_{T451} -PKR-KD activation was not significant between D0 and M24, it reached 74.3%.

Western blots were performed to determine the expression of total PKR (74kDa) and its phosphorylated forms: full-length P_{T451} PKR (74kDa) and P_{T451} -PKR-KD (35kDa) as described in method section. Protein expression level was reported to the actin expression level (42kDa) and expressed in arbitrary units. Activation of full-length PKR was calculated using the ratio between full-length P_{T451} -PKR and total PKR. Activation of PKR-KD was calculated using the ratio between P_{T451} -PKR-KD and total PKR. Representative blots showed protein expression in PBMCs of the same patient at D0, M12 and M24. A Friedman's test showed significant differences for total PKR ($p=0.0001$), full-length P_{T451} PKR ($p=0.0247$) and P_{T451} -PKR-KD activation ($p=0.0053$) during the follow-up. A Scheffé's post-hoc test indicated $p<0.05$ between D0 and M12 for the levels of total PKR expression.

Correlation of PKR and activated PKR at baseline with cognitive decline

We found a correlation between total PKR expression at baseline and absolute variation of MMSE at 6 months ($r=0.34$, $p=0.0497$). We found a correlation between PKR-KD activation at baseline and cognitive decline at 6 months with absolute and relative variation of ADAScog ($r=0.33$, $p=0.0506$ and $r=0.33$, $p=0.0453$, respectively).

PKR and its activation of full PKR or PKR-KD strongly correlated with cognitive decline at one year (Table 2). No correlation was found with P_{T451} -PKR or P_{T451} -PKR-KD. We found a correlation between PKR-KD activation and relative variation of ADAScog at two years ($r=0.36$, $p=0.0407$). Among the three parameters studied at baseline: total PKR expression, full-length PKR activation and PKR-KD activation, only the activation of PKR-KD was correlated with each assessment of cognitive score (6, 12 and 24 months). This activation could be then a good predictive biomarker of the cognitive decline in AD. No correlation was found between PKR and its activation at M12 and cognitive decline between M12 and M24 assessed by ADAScog or MMSE.

Discussion

In this longitudinal study, results show that total PKR levels and the activation of this kinase in PBMCs are associated with individual cognitive decline at one year in newly diagnosed patients with AD at an early stage (median MMSE score at 20.5) and at two years with a lower correlation. Previous studies report a negative individual correlation between full length PKR activation and cognitive status in patients at an advanced stage with a medium MMSE score of 15.4 points [18]. Dumurgier et al. reported that a higher level of phosphorylated PKR in CSF at the T446 site was associated with a more marked decline in patients with a medium MMSE score of 20.5 at baseline [17]. We report for the first time individual correlation of PKR activation in PBMCs with

	Total PKR	Full PKR activation	PKR-KD activation
AV ADAScog	$r=-0.4$; $p=0.0134$	$r=0.45$; $p=0.0051$	$r=0.40$; $p=0.0126$
RV ADAScog	$r=-0.49$; $p=0.0024$	$r=0.54$; $p=0.0009$	$r=0.48$; $p=0.0033$

Correlations were performed using Spearman correlation coefficient (rho) calculation, level of significance was $p<0.05$.

Table 2: Correlation between PKR and its activation with ADAScog Absolute Variation or Relative Variation (AV and RV) at one year

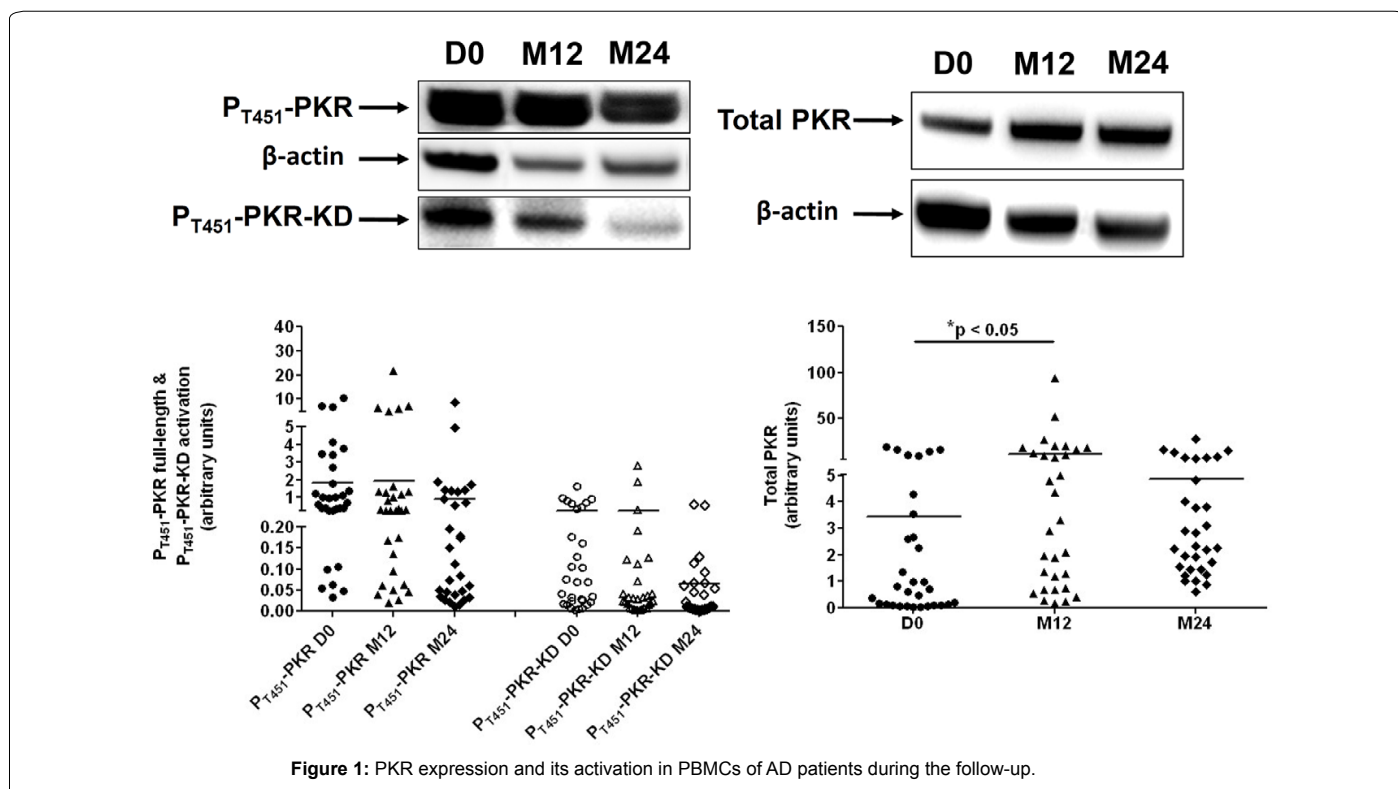


Figure 1: PKR expression and its activation in PBMCs of AD patients during the follow-up.

individual cognitive decline. We chose to assess PKR phosphorylation at the T451 site because it is one of the first sites of phosphorylation, using custom antibodies which have shown their reliability in previous studies [9,18,27].

We assessed expression of PKR and its phosphorylated forms using western blot technique with results expressed in arbitrary units. These data were used to calculate PKR activation. Statistical analysis did not show significant differences during the two years follow-up excepted for total PKR expression which was increased at M12 compared to D0. However, a great variability of values was observed for the PKR expression by western blot, limiting other significant differences with non-parametric statistical tests. Moreover western blot quantifies only the relative expression of proteins. It would then be interesting to develop an ELISA test to determine the absolute quantity of PKR and its phosphorylated forms.

To our knowledge, this is the first longitudinal study of PKR activation in the context of AD, as previous reports were transversal. Our results show a strong correlation between total PKR expression or its activation and cognitive decline assessed by ADAScog but less correlation with MMSE. An explanation could be that ADAScog scale, which is often used as primary endpoint in randomized trials, is a more global scale with a larger range. MMSE may be less sensitive to identify light cognitive variation. The strongest correlation was found with cognitive decline after one year. Furthermore, the activation of PKR-KD was highly correlated with absolute and relative ADAScog variation at all assessments M6, M12 and M24, indicating that this ratio of P_{T451} -PKR-KD/total PKR could be a good predictive biomarker in AD. With the evolution of the disease, the severity of the cognitive degradation could no longer reflect the progression of the disease. Our results suggest an early involvement of PKR which is stable over time followed by other mechanisms like inflammation and autophagy, because PKR has many downstream targets.

Thus PKR activation can predict cognitive decline by its implication in several pathways. Beside amyloid plaques and neurofibrillary tangles, inflammatory process is involved in AD [28]. PKR plays a critical role in the control of pro-inflammatory cytokine production [29] particularly in the context of AD [9]. PKR activation may also promote neuronal death by down regulation of translation and pro apoptotic effects [30].

Our study has some limitations. First, all patients were included in the solo center of Poitiers and the number is limited. PKR and its activation need to be compared with other biomarkers such as brain atrophy or CSF markers. Similarly a comparison of PKR activation in PBMCs and in CSF should be done.

Conclusion

Our results show that PKR activation negatively correlates with cognitive decline in newly diagnosed AD patients at an early stage of the disease. The PKR-KD activation could be used as an early pronostic marker but our results need to be confirmed in a larger cohort and assessment of PKR should be performed using ELISA technique that requires to be developed directly into PBMCs without culture.

Déclarations

Ethics approval and consent to participate: All patients signed a written informed consent. This study was approved by the local ethic committee (comite de protection des personnes Ouest 3) and declared on ClinicalTrial.gov.NCT01351142.

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