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Combination of p53(ser15) and p21/p21(thr145) in peripheral blood lymphocytes as potential Alzheimer's disease biomarkers

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ABSTRACT

Alzheimer's disease (AD) is still difficult to be precisely diagnosed in its early stage to date. Establishing of reliable and manageable disease-specific biological markers is required to improve diagnostic accuracy. Based on the hypothesis of cell cycle regulatory failure at the early stage of AD, we tested whether cell cycle regulating proteins p53, p21 and their phosphorylated forms p53(ser15), p21(thr145) were changed in AD patients and whether these proteins could be used as diagnostic biomarkers. Western bolt, Enzyme-linked immunosorbent assay (ELISA), immunofluorescent staining and flow cytometry (FCM) analysis were employed to analyze levels of these proteins in peripheral blood lymphocytes (PBLs) from 95 controls, 94 AD, 12 Parkinson's disease (PD) and 15 vascular dementia (VaD) patients. Compared with controls, p53(ser15) and p21(thr145) levels were significantly increased and p21 level was significantly decreased in PBLs of AD patients but not in PD or VaD, while p53 was increased in both AD and VaD patients. The receiver operating characteristic (ROC) curve analysis showed that the specificity and sensitivity were 76% and 84% for p53, 88% and 82% for p53(ser15), 80% and 75% for p21 and 84% and 68% for p21(thr145) in identifying AD patients. The relatively high diagnostic accuracy support these proteins, especially p53(ser15) and p21 in PBLs may become potential biomarkers for diagnosis of AD.

1. Introduction

Alzheimer's disease (AD) has become one of the leading causes of disability with enormous socioeconomic costs. Early and precise diagnosis of AD is very necessary and a basis for medical intervention to improve the patient's quality of life [7]. Since there is a long duration of pathophysiological changes before an AD patient shows cognitive loss and symptoms required for clinical diagnosis appear after years or decades of the disease process [11], exploring valid, easy and accessible biomarkers in its early stage is very needed [3,4].

The use of peripheral blood lymphocytes (PBLs) as potential diagnostic tool for early diagnosis of AD stems from the hypothesis of cell cycle regulatory failure of neurodegenerative diseases. The aberrant of cell cycle may be the earliest neuropathological event detected in AD so far, as it plays a crucial role from beginning [13]. Direct evidence supports that cell cycle regulatory failure is not only limited to neurons in AD patients, but also takes place in peripheral cells, such as lymphocytes [6].

The failure of cell cycle control in AD has been identified to occur at cell cycle "G1/S transition checkpoint" [18], which is regulated by p53-p21 signaling pathway [19]. Tumor suppressor protein p53 is nuclear phosphoprotein whose normal function is to regulate cell cycle and apoptosis [17]. Cyclin-dependent kinase inhibitor p21 is a p53-inducible protein, which mediates p53-dependent cell cycle arrest [10]. Meanwhile, phosphorylation of p53 and p21 is one of the important mechanisms that regulate their stability and protein levels. An increase in p53 level has been found in PBLs from AD patients [9,25]. Our present study is to determine whether the levels of p53, p21 and their phosphorylated forms p53(ser15) and p21(thr145) are changed in PBLs of AD patients and whether these proteins could become potential biomarkers for diagnosis of AD.

2. Materials and methods

2.1. Subjects

In total, 95 controls, 94 AD, 12 PD and 15 VaD patients were recruited from Xuan Wu Hospital, Beijing Geriatric Hospital and Huairou Community in Beijing. The clinical diagnosis of the probable AD was based on the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorder Association

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(NINCDS-ADRDA). None of the AD patients had other neuropsychiatric disorders and family history of AD. Age-matched healthy controls were subjects without clinical signs of neurological or psychiatric diseases. VaD and PD patients were diagnosed according to NINDS-AIREN criteria [20] and UK Parkinson's Disease Society Brain Bank criteria [15] respectively.

All subjects were examined and diagnosed based upon interview, physical examination, blood tests, brain MRI and neuropsychological assessment by at least two senior neurologists. Cognitive status was evaluated using mini mental state examination (MMSE) and clinical dementia rating (CDR). None of the subjects chosen in this study suffered from infection, neoplastic or autoimmune disease when the peripheral blood samples were collected. Informed consent was acquired from all subjects or from their caregivers and the protocol of this study was approved by the Ethical Committee of the Capital Medical University.

2.2. Samples processed

The heparinized peripheral blood (5 ml) was collected, and lymphocytes were isolated by Ficoll Hypaque. After washing in phosphate-buffered saline (PBS), cells protein was extracted as previously described [22] and stored at −80 °C in plastic vials. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, USA). In addition, some lymphocytes were suspended in RPMI-1640 medium, containing fetal bovine serum (10%, v/v), penicillin (100 μg/ml), streptomycin (100 μg/ml), and cultured in a humidified 5% CO_2 incubator at 37 °C and used for immunofluorescent staining and flow cytometry (FCM) analysis.

2.3. Western blot analysis

Equal amount (60 μg) of whole cell extracts were electrophoresed on 12.5% SDS polyacrylamide gels, and then transferred to nitrocellulose filter membranes (PALL, USA). The membranes were blocked with non-fat milk and incubated at 4 °C overnight, with the primary antibodies from Santa Cruz at the following dilutions: mouse anti-p53 1:250, rabbit anti-p21 1:250, rabbit anti-p53(ser15) 1:200, rabbit anti-p21(thr145) 1:200 and mouse anti-β-actin 1:500. After washing, the membranes were appropriately incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:1000; Santa Cruz, USA). Signals were detected with a chemiluminiscent substrate detection system Lighten® HRP-B (Viagene, CN). The relative protein levels were determined by scanning and measuring the bands with FluorChem Imaging Analysis System (Alpha Innotech, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA) analysis

Protein samples were diluted to a final concentration of 20 μg/ml in PBS. Each diluted sample (50 μl) was coated onto 96-well plates. After washing, coated wells were blocked in PBS containing 1% BSA and 0.05% Tween-20 for 2 h and then incubated with primary antibodies (anti-p53 1:100; anti-p21 1:200; anti-p53(ser15) 1:100; anti-p21(thr145) 1:200; anti-β-actin 1:500; Santa Cruz) at 4 °C overnight. Antibody binding was visualized using peroxidase-conjugated secondary antibody (1:1000; Santa Cruz). Color was developed by TMB (Beyotime, China) as a substrate and reaction was stopped by 2 M H_2SO_4. Optical density (OD) was determined at 450 nm with PARADIGM™ Detection Platform (Beckman Coulter, USA). The protein level was expressed as the ratio between the OD of interest protein and β-actin of the same sample. Measures were made in duplicate and inter assay variability was less than 9%.

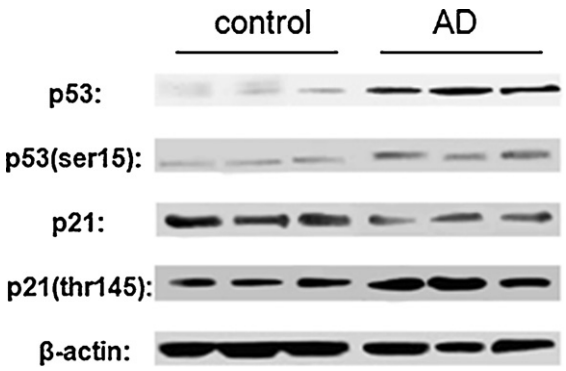

Fig. 1. Immunoblotting of p53, p53(ser15), p21 and p21(thr145) in PBLs from AD patients and controls. The bands of these proteins and β-actin are shown.

2.5. Immunofluorescent staining and FCM analysis

The cultured PBLs were fixed in 1% formaldehyde in PBS for 15 min. After washing, cells were permeabilized with 0.2% Saponin (Sigma, USA) in PBS solution for 1 h and then incubated at 4 °C overnight, with the primary antibodies just mentioned above (1:50 in PBS/1% BSA solution), recognizing p53, p21, p53(ser15) and p21(thr145) respectively. Cells rinsed in PBS were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:50 in PBS/1% BSA). After rinsing, 20 μl of cell suspension were deposited on glass slides and observed at ×40 magnification using an inverted fluorescence microscope Olympus TH4-200 (Olympus, Japan). Cell suspensions were also analyzed with a flow cytometer (Beckman Coulter, USA). Lymphocytes population was identified by forward and side-angle scatter and interest protein emissions were detected in the FL-1 channel (525 nm). For each sample, data from 30,000 events were recorded in list mode, displayed on logarithmic scales and analyzed using Expo32 v1.2 software.

2.6. Statistical analysis

All experimental data were expressed as mean ± SD and analyzed using SPSS 13.0 statistical software. Following analysis of variance, differences between groups were estimated using independent samples *t*-test and one-way analysis of variance (ANOVA). Sensitivity and specificity of measured variables for the diagnosis of AD were determined by receiver operating characteristic (ROC) curve analysis using a nonparametric approach. The best cut-off values were selected as those which minimize the sensitivity-specificity difference and maximize discriminating power of the tests. Significance was considered at $P < 0.05$.

3. Results

Firstly, Western blot analysis (Fig. 1) was used to measure the relative levels of p53, p21, p53(ser15) and p21(thr145) in PBLs from 10 AD patients and 10 controls (Table 1a). The ratios of interest protein to β-actin revealed significant increase in the levels of p53, p53(ser15), p21(thr145) ($P < 0.01$) and a decrease of p21 ($P < 0.05$) in AD patients compare to controls.

Then, another group of 10 healthy controls and 10 AD patients was collected (Table 1b) for immunofluorescent and FCM analysis (Fig. 2). Compare to control cells, AD lymphocytes showed significantly high fluorescence intensity of labeled p53, p53(ser15), p21(thr145), and low fluorescence intensity of labeled p21. Quantitative FCM analysis was used and significant differences were detected in the percentage of positive lymphocytes between AD patients and controls: p53 (AD: 68.76 ± 3.34; control: