"Searching for predictive blood biomarkers: misfolded p53 in mild cognitive impairment."

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**Abstract**

The identification and validation of biomarkers for preclinical patients with mild cognitive impairment (MCI) at-risk for Alzheimer's disease (AD) development is increasingly important. We used the cytofluorimetric analysis of unfolded p53 to determine the prognostic ability of the protein as predictive signature from MCI to AD in a longitudinal study of a population of presymptomatic patients with the clinical diagnosis of MCI. Venous blood samples from 24 healthy subjects, 28 MCI and 15 AD were analyzed with the cytofluorimetric method for unfolded p53 protein detection. Twenty-four MCI patients had clinical follow-up subsequent to the analysis for unfolded p53. Elevated levels of the conformationally altered protein were able to discriminate both MCI and AD patients comparing with healthy subjects. Longitudinal follow-up revealed that 7/24 MCI patients progressed to AD. All converters (100%) were predicted by elevated levels of unfolded p53, with a positive predictive value of 87....

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Searching for Predictive Blood Biomarkers: Misfolded p53 In Mild Cognitive Impairment

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Abstract: The identification and validation of biomarkers for preclinical patients with mild cognitive impairment (MCI) at-risk for Alzheimer’s disease (AD) development is increasingly important. We used the cytofluorimetric analysis of unfolded p53 to determine the prognostic ability of the protein as predictive signature from MCI to AD in a longitudinal study of a population of presymptomatic patients with the clinical diagnosis of MCI.

Venous blood samples from 24 healthy subjects, 28 MCI and 15 AD were analyzed with the cytofluorimetric method for unfolded p53 protein detection. Twenty-four MCI patients had clinical follow-up subsequent to the analysis for unfolded p53. Elevated levels of the conformationally altered protein were able to discriminate both MCI and AD patients comparing with healthy subjects. Longitudinal follow-up revealed that 7/24 MCI patients progressed to AD. All converters (100%) were predicted by elevated levels of unfolded p53, with a positive predictive value of 87.5%.

These data support and extend our previous observation that the cytofluorimetric approach for unfolded p53 protein was able to discriminate AD patients from healthy subjects and to predict the progression from MCI to AD in presymptomatic patients before clinical diagnosis for AD was evident.

Keywords: Alzheimer’s disease (AD), blood biomarker, flow cytometry, mild cognitive impairment (MCI), unfolded p53, positive predictive value.

INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia of the elderly population and a major public health problem [1]. Currently, there is no disease-modifying cure for AD because of an incomplete understanding of its pathogenesis. The major goal of AD research is the prevention of the disease, which entails early detection before the onset of symptoms. The degenerative process in AD probably starts 20-30 years before the clinical onset of the pathology [2]. This preclinical phase lies within the boundaries of the diagnosis of mild cognitive impairment (MCI). MCI is an evolving diagnostic construct which is usually defined by the presence of subjective and/or objective cognitive complaints in elderly subjects which are not at the late stages corresponding to dementia. MCI subjects show a measurable memory disorder, decline in objective memory performance but are not demented and do not have or have minimal changes in activities of daily living [3]. As such, MCI is defined as a transition stage between the cognitive decline of normal aging and full-blown AD [4]. Individuals with MCI constitute a high-risk population; in a number of longitudinal studies this designation carries a high risk for the development of dementia with conversion rates in the range of 5-15%/year [5-8]. Although subjects with MCI may have an increased risk to develop AD, this clinical state encompasses several subtypes of cognitive dysfunctions of different etiologies, none of which necessarily progress to AD [9].

The current inability of clinical criteria to accurately identify this at-risk group for AD development is fuelling the interest in biomarkers aimed to completing clinical approaches. Within this reference frame, we studied p53 in aged controls and demented patients finding the existence of a conformational state of p53 protein in blood cells that allows to distinguish AD patients from control subjects and patients affected by other dementias [10].

Recently, we showed for the first time that the blood-based cytofluorimetric assay of unfolded p53 can be used as an easy accessible adjunctive diagnostic tool in identifying those MCI patients from the at-risk group who progress to AD [11].

In this context, the aim of the present study was to validate the capability of unfolded p53 to predict progression to AD in a longitudinal study on an independent population of presymptomatic patients with MCI.

MATERIAL AND METHODS

Study Groups

We studied a group of subjects consisting of 15 patients with sporadic AD, 28 patients with MCI and 24 healthy age-
matched controls (CTR) (Table 1). Venous blood samples were obtained from the Institute "Fondazione Casimiro Mondino" in Pavia, Italy. The study, including the follow-up visits, was approved by the Ethical Committee and a written consent was obtained from all subjects or, where appropriate, their caregivers.

All the subjects were examined by a senior neurologist and diagnosis of dementia was made according to DSM-IV and the NINCDS-ADRSA criteria. All MCI subjects met the original Petersen/Mayo criteria for MCI [12, 13]. Dementia was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT Scan and MRI) investigation. Cognitive status was quantified using the Mini Mental State Examination (MMSE) and with a neuropsychological battery of tests assessing learning and episodic memory, speed and attention, language and visuo-spatial functions. Neuroimaging studies were included as part of the diagnostic setup; all patients underwent a CT scan. The presence/absence of temporal atrophy was determined by a categorical variable measuring the radial width of the temporal horn and taking into consideration the cut-off values for CT images [14]. The presence/absence of white matter alterations was also considered; to differentiate AD from vascular dementia we excluded those patients who presented with a white matter involvement of 25% or more [15]. Control subjects were individuals with no clinical signs of neurological or psychiatric diseases, mostly enrolled among spouses of the MCI and AD group of patients. None of the subjects selected in this study was affected by neoplastic or autoimmune disease when the blood samples were taken. For each subject the count of leukocytes was within the regular reference interval. The progression to AD was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, CT Scan and MRI investigations.

Flow-Cytometry

Blood samples (3-4 ml) were collected by venipuncture and diluted in PBS solution 1% to the final ratio of 1:3. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll Hypaque density gradient from Na+/citrate samples (Eurobio Laboratories, Paris, France) and fixed in 2% formaldehyde in phosphate buffered saline (PBS). Rinsed cells were permeabilized with 0.2% saponin in PBS solution and incubated in ice for 30 min with a primary monoclonal antibody recognizing mutant p53 (clone PAb240; NeoMarkers, Fremont, CA, USA) (4 mg/ml in PBS/1% bovine serum albumin (BSA) solution). Cells rinsed in PBS/1% BSA were incubated for 30 min in ice with a goat anti-mouse immunoglobulin (lg) G antibody phycoerythrin-conjugated (DakoCytomation, Glostrup, Denmark; 1:40 in PBS/1% BSA). Cell suspension was then analyzed with a flow cytometer Partec PASII (Partec, GmbH, Munster, Germany). PBMC population was identified by forward and side-angle scatter and mutant p53 emission was detected in the FL-2 channel (535–580 bandpass filter). For each sample, data from 20 000 events were recorded in list mode, displayed on logarithmic scales and analysed using WinMDI 2.8 software.

Molecular Genetic Analysis

Genomic DNA was extracted from peripheral leukocytes with a commercially available nucleic acid purification kit (Wizard DNA Purification Kit, Promega). The Apolipoprotein E (APOE) gene polymorphisms were determined by Hha I restriction endonuclease digestion of PCR products, according to Hixson and Vernier [17].

Statistical Analysis

The data were analyzed using GraphPad Prism software by analysis of variance (ANOVA) followed when significant by the Bonferroni’s Multiple Comparison Test. Differences were considered significant when a p-value ≤ 0.05 was attained.

RESULTS

67 subjects were enrolled among those referring to the Institute "Fondazione Casimiro Mondino" in Pavia, Italy. Control subjects, MCI and AD patients were comparable as far as age and gender distribution (see Table 1). The memory impairment evaluated by the Mini-Mental State Examination (MMSE) showed a greater deficit in AD patients compared to MCI and healthy controls subjects. 4 of the 28 MCI patients and 11 of the 15 AD patients presented medial temporal (MT) atrophy on CT scan and MRI that was performed at the enrollment. The distribution of e2/e3/e4 alleles of APOE was 0.100/0.665/0.235 in AD cases and 0.040/0.960/0.0 in control subjects; the frequency of the APOE e4 allele was, as expected, higher in AD population than in control subjects. MCI patients showed a distribution of 0.020/0.840/0.140 (see also Table 1 for genotype distribution).

Blood mononuclear cells derived from healthy subjects, MCI and AD patients were subjected to cytofluorimetric analysis by using the conformational specific antibody PAb240, which discriminates unfolded p53 tertiary structure. A highly statistically significant difference was found in the percentage of PAb240 positive cells when comparing controls with patients affected by MCI or AD patients (percentage of PAb240 positive cells, mean ± standard deviation; control subjects: 28.6 ± 11.8, MCI: 38.4 ± 12.7, AD: 43.4 ± 18.8; MCI versus control P < 0.05, AD versus control P < 0.01) (Fig. 1).

Focusing on the MCI group (28 patients), we found that 6 of them expressed APOEe4 allele and 4 presented MT atrophy on CT scan and MRI. In addition, the measurement of the percentage of PBMC expressing unfolded p53 of MCI patients defined two well-separated groups. The division in two groups was made considering one standard deviation over control mean (calculated reference value 40.4%), consistently with other previous data from literature [18].

The first group (n = 15) had percentages of unfolded p53 values ranging from 20% to 38% and the other group (n = 13) expressed values ranging from 40% to 63.3%. The latter was defined as candidate at high risk to develop AD. Thus, according to the predicted criteria, a total of 9 patients (6 with APOE e4, 4 with brain atrophy, 9 with high unfolded p53) were considered to bear one or more risk factors putting them at high risk to develop AD.
Table 1. Demographic and Clinical Variables and Genotype Frequency of the APO-E Polymorphisms of all the Subjects. N: number; M: male; F: female; L.O.I.: length of illness; MMSE: Mini-Mental State Examination. Data are expressed as mean ± standard deviation. For the genotype frequency values are expressed as number (%).

<table>
<thead>
<tr>
<th></th>
<th>CTR</th>
<th>MCI</th>
<th>AD</th>
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<tbody>
<tr>
<td>N (M:F)</td>
<td>24 (12;12)</td>
<td>28 (11;17)</td>
<td>15 (6;9)</td>
</tr>
<tr>
<td>Age</td>
<td>71 ± 6</td>
<td>70 ± 7</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>L.O.I. (month)</td>
<td></td>
<td>28 ± 13</td>
<td>34 ± 11</td>
</tr>
<tr>
<td>MMSE</td>
<td>29 ± 1</td>
<td>26 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Medial-Temporal atrophy (Yes;No)</td>
<td>24 (0;24)</td>
<td>28 (4;24)</td>
<td>15 (11;4)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ε2/ε3</td>
<td>2 (8)</td>
<td>1 (4)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>ε3/ε3</td>
<td>22 (92)</td>
<td>21 (75)</td>
<td>6 (40)</td>
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<td>1 (7)</td>
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<tr>
<td>ε4/ε4</td>
<td>0</td>
<td>2 (7)</td>
<td>0</td>
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Fig. (1). Box plot reporting the amount of conformationally altered p53 (%) expressed in peripheral blood cells for each patient enrolled in this study. Patients are divided according to the diagnostic group. p53 protein expression in blood cells from control subjects, AD and MCI patients. Bonferroni’s Multiple Comparisons Test has been used for statistical analysis. * p < 0.05 and ** p < 0.01 vs control.

After a mean of 15 ± 3 months from the recruitment in the overall study population, 4 MCI patients were lost because they did not report to secondary screening and 7 over 24 MCI subjects progressed to AD. All converters were predicted by high levels of unfolded p53, with a positive predictive value (PPV) of 87.5% that is comparable with data reported by Zetterberg et al. [19] on combined CSF biomarkers. Among converted patients, four expressed the APOEε4 variant and four presented MT atrophy on MRI. Among the non-converted group (17 patients), two expressed APOEε4 variant, none had MT atrophy and two had high unfolded p53 (Fig. 2).

Moreover, dividing patients according to the new diagnosis at the follow-up, the amount of unfolded p53 expressed in peripheral blood cells, analyzed at the enrollment, still showed a highly statistically significant difference in the percentage of PAb240 positive cells when comparing converted patients and AD patients with controls and not-converted MCI (percentage of PAb240 positive cells, mean ± standard deviation (SD); control subjects: 28.6 ± 11.8; not-converted: 34.5 ± 11.9; converted: 50.1 ± 6.1; AD: 43.4 ± 18.8; converted and AD versus control subjects P < 0.01, converted versus not-converted P < 0.05) (Fig. 3).

**DISCUSSION**

The current work explored the use of blood unfolded p53 measurement in a cohort of preclinical patients with MCI as predictive signature to AD conversion. Our central goal was to extend the limited literature of unfolded p53 expression in this population.

In this research field, measurement of p53 conformational status in blood cells has been found to discriminate AD cases from normal ageing, Parkinson’s disease and other dementias [2, 20]. In particular, the measurement of conformationally altered p53 has been demonstrated to be highly sensitive mainly in young patients with a sensitivity of 90% in subjects below 70 years of age [10]. Since, p53 conformational changes found in AD cells have been demonstrated to be independent on gene mutations [21], it is reasonable to conceive that the mechanism by which p53 changes its conformational state is linked to the pathogenic events occurring in AD. A pathogenic link between p53 conformational
changes and AD was suggested indirectly by the effects of soluble low concentrations of beta-amyloid on p53 tertiary structure in fibroblasts [22] and other cell lines [23, 24]. A growing body of evidence suggest that p53 may play a role in different neurodegenerative diseases, such as Parkinson’s disease (PD) [25] and amyotrophic lateral sclerosis (ALS) [26]. Specifically concerning AD we and others demonstrated that the unfolded isoform of p53 in fibroblasts [23] and in other cell lines [24, 25] can be induced by nanomolar concentrations of beta-amyloid. The consequence of the presence of unfolded p53 was reported to be responsible of alterations in the molecular machinery involved in maintaining neural function in neurodegenerative disease and influence the signaling pathways regulating the balance of cell survival versus death, a decision often governed by checkpoint proteins [27]. As an example unfolded p53 seems to be involved in the failure in the G1/S transition checkpoint in lymphocytes of AD patients [28] and the causes of such a conformational change could be oxidative stress. Furthermore it has been observed that transgenic mice model of AD overexpressing mutations for both APP and presenilin-1 (PS1) show an important increase of unfolded p53 compared to wild-type littermates [29].

These additional observations about the existence of an altered tertiary structure of p53 at the central and peripheral level in both animal models and subjects with AD reinforce the hypothesis that the protein can have a role in the pathogenesis, but further studies are still required to understand in detail the causes and consequences of such a conformational change.

In our study, to assess whether p53 conformational changes are an early event in AD pathology we followed up patients falling in the category of MCI for 15 ± 3 months. Analysis of unfolded p53 was performed at the beginning of the enrollment and was used to predict which subjects of MCI patients will progress to AD.

![Fig. (2). Scheme of converted and not-converted MCI patients based on unfolded p53 analysis and APOEe4 genotype. Twenty-eight MCI (11 males and 17 females) with a mean ± standard deviation age of 70±7 and MMSE score of 26±2 were examined. Cognitive status was quantified using the Mini Mental State Examination (MMSE). None of the subjects selected in this study was affected by neoplastic or autoimmune disease when the blood samples were taken. Patients were divided in four groups according to MT atrophy, APOEe4 variant and high unfolded p53. 15 months later, patients were re-evaluated and classified as converted and non-converted in AD (A, B).](image1)

![Fig. (3). Box plot reporting the amount of conformationally altered p53 (%) expressed in peripheral blood cells of patients enrolled in this study. Patients are divided into four groups: p53 protein expression in blood cells from control subjects, AD and MCI patients, converted and not-converted to AD after reanalysis 1.5 years following enrollment and p53 measurement. Bonferroni’s Multiple Comparisons Test has been used for statistical analysis. ** p < 0.01 vs control and # p < 0.05 vs not converted.](image2)

The results obtained in the present study confirm and extend our previous observation on AD and MCI patients, by finding that the cytofluorimetric approach for unfolded p53 protein was able to discriminate AD patients from healthy subjects and to predict the progression from MCI to AD in a small cohort of MCI patients before clinical diagnosis for AD was evident. The rate of progression of MCI to AD was
comparable with the mean reported in population studies [18, 29]. In fact we found that 29% of MCI patients converted to AD after 15 ± 3 months from the recruitment. The high expression of unfolded p53 may be considered as high risk factor for the conversion to AD since in the MCI converted group the 100% of converters was predicted based on elevated levels of unfolded p53, whereas only 57% was predicted based on APOE status and MT atrophy. Altogether these results confirmed that elevated levels of unfolded p53 might be considered a biomarker for AD and a high risk factor for the conversion from MCI to AD.

The search for predictive biomarkers for AD is a high priority in neurodegenerative disease research. Biomarker development for AD currently encompasses six different but inter-related approaches: (i) behavioral assessment of the patient including measurements of cognitive status [30, 31] that include the MMSE [32]; (ii) assessment of changes in brain volume [33]; (iii) alterations in brain metabolism [34]; (iv) measurement of beta-amyloid load within the brain [33]; (v) CSF biomarker profiles [35] and (vi) post-mortem confirmation of AD histopathology. The utility of these various biomarker approaches, individually or collectively, has yet to be established. It is becoming clear that the evaluation of a single biomarker might not be potent enough to improve diagnostic specificity to distinguish one pathology fingerprint from other disease profiles. In the search of a patient-specific, multi-biomarker profile, at present more likely a combination of cerebrospinal fluid (CSF) biomarkers [36] seems to have a higher likelihood of success allowing sensitive and specific diagnosis of AD. Unfortunately, the use of CSF biomarkers is limited because of invasive sample collection methods. Efforts are underway to discover reliable blood biomarkers. To date, it seems probable that only the combination of several biomarkers derived from blood will be successful to define a disease-specific signature. In this reference frame, search of biomarkers in the blood compartment has seen the significant contribution of the groups directed by Wyss-Coray. They developed a proteomic analysis of 18 proteins in the plasma of patients able to predict with 90% accuracy the diagnosis of AD [37]; furthermore these results confirmed that elevated levels of unfolded p53 might be considered a biomarker for AD and a high risk factor for the conversion from MCI to AD.

Since it is essential to identify a battery of determinations to increase the accuracy of the diagnosis, we also evaluated in p53-positive converters the additional presence of a number of biomarkers as potential predictors of conversion to AD in MCI populations. Characteristics that make patients more “AD-like” have been associated with a higher rate of development of clinical AD. For example, hippocampal atrophy [40], low cerebrospinal fluid (CSF) amyloid-beta (Aβ) and elevated tau [41, 42], and the presence of the apolipoprotein ε4 (APOE ε4) allele [43] predicted a higher rate of conversion to AD. Within this reference frame, we found the presence of APOE ε4 allele and a significant atrophy in the medial temporal lobes of unfolded p53-positive MCI patients, consistent with numerous studies reporting APOE ε4 [43, 44] and medial temporal volumes as a predictor of conversion to clinical AD in a-MCI populations [40]. We suggest that the absence of these three factors may be considered as a positive prognosis for the conversion to AD.

However, it is worth to note that also among the non-converted group (17 patients) two patients expressed APOEε4 variant and two had high unfolded p53. We cannot rule out that probably these four MCI patients at high risk of conversion will convert in the future according to the rate of progression to dementia documented in literature [18].

CONCLUSION

Here we found that high values of unfolded blood p53, which has been linked to AD pathology, may be considered as high risk factor for the conversion to AD, with a positive predictive value of 87.5% that is comparable with data on combined CSF biomarkers. We are aware that the sample size of our study is too small to reach a definitive conclusion and the definition of conformationally altered p53 as predictive signature from MCI to AD requires a further investigation in larger populations of patients. We suggest that measurement of conformational p53 state can be useful as an easy accessible adjunctive diagnostic tool in identifying those MCI patients who are at risk to progress to AD.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MCI</td>
<td>Mild Cognitive Impairment</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>CTR</td>
<td>Controls</td>
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<td>CT Scan</td>
<td>Computerized Tomography</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>MMSE</td>
<td>Mini Mental State Examination</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>FSC</td>
<td>forward angle scatter</td>
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<tr>
<td>SSC</td>
<td>side angle scatter</td>
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<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>MT</td>
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</tr>
<tr>
<td>Aβ</td>
<td>beta-amyloid</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>PPV</td>
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REFERENCES


