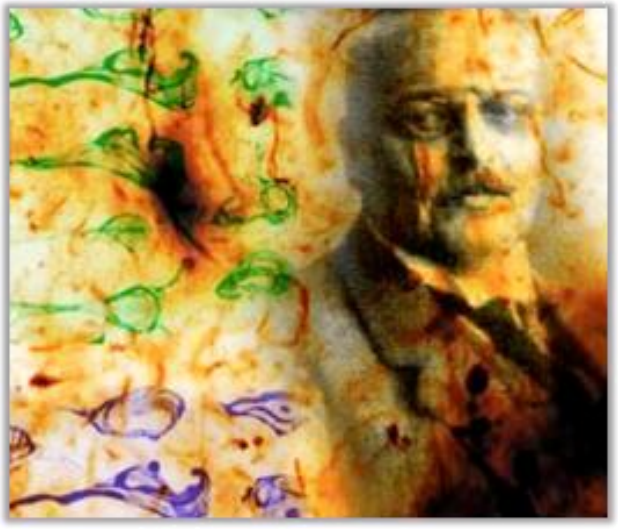
UNIVERSITY OF PAVIA

Department of Experimental and Applied Pharmacology

"p53 in Alzheimer's disease: from biomarkers to molecular mechanisms"



Serena Stanga

Ph.D. in Pharmacological Sciences

XXIII Cycle - A.Y. 2007-2010

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Preface

I spent my Ph.D. research in Pharmacological Sciences in the laboratory of Neurobiology and Neuropharmacology of Aging and Neurodegeneration in the Department of Experimental and Applied Pharmacology of the University of Pavia.

During my research, I focused both on diagnostic and molecular aspects of Alzheimer's disease (AD) with the aim to find a biomarker for the early detection and to understand the pathogenic mechanism underlying the disease.

AD is a progressive neurodegenerative disorder characterized by irreversible cognitive and physical deterioration that commonly begins with loss of recent memory, follows by a progressive cognitive decline and finally dementia. The hallmark pathological lesions for the confirmatory diagnosis are amyloid plaques and neurofibrillary tangles together with synaptic loss.

Due to the aging of the population, AD is a growing socioeconomic and public health problem because its incidence doubles every five years between 65 and 85 years of age and it has major consequences on the life of the patient and his/her caretaker and its cost is important. Actually, there is no cure for the disease because of an incomplete understanding of the events that lead to the selective neurodegeneration of AD brain.

The degenerative process probably starts 20–30 years before the clinical onset of the disease and this clinical phase, between

prodromal and established AD, is defined mild cognitive impairment (MCI). One of the major goal of AD research in the present era is the prevention of the disease, which entails early detection before the onset of symptoms. For this reason MCI, the earliest clinically detectable phase of dementia and AD, has become a focus of clinical, epidemiologic, neuroimaging, biomarker, neuropathological, disease mechanism and clinical trials research.

As reported in the first Chapter of this thesis we have identified that an altered conformation of a protein named p53, constitutively expressed to maintain genomic integrity, is a new predictive biological marker of transition from MCI to AD.

In Part 1, the focus is on peculiar aspects of pharmacogenetic and pharmacogenomics research in aging and age-associated diseases to define novel targets for therapeutic interventions aimed to checkpoint proteins. In fact, aging in higher animals may be influenced by the balance of cell survival versus death, at least in part regulated by a fine timing of checkpoint proteins and preservation of DNA integrity and correct repair. p53 is one of this proteins, recently shown to be involved in aging and AD.

In Part 2, using peripheral blood cells from MCI and AD patients, we describe a blood-based cytofluorimetric method for unfolded p53 protein detection that predicts the progression to AD in preclinical patients with MCI two years before clinical diagnosis of AD was made and we discuss the potential of the test in diagnosing AD.

In Part 3, I describe my recent results concerning the study of unfolded p53 as marker of progression from MCI to AD. In

particular, I confirm in a longitudinal study on an independent population of patients, that elevated levels of unfolded p53 may be considered as high risk factor for the conversion to AD.

Moving from the need of a biological marker to improve diagnostic accuracy, the second Chapter of this thesis is focused on the study of the molecular mechanism underlying the pathogenesis of AD. Previously, we found that the exposure to nanomolar concentrations of the amyloid peptide (Aβ) was responsible for an increased content of unfolded p53 in fibroblasts from control subjects; for this reason we hypothesis that low amounts of soluble Aβ can induce early pathological changes at cellular level that may precede the amyloidogenic cascade. One of these changes is the induction of the unfolded state of p53, suggesting a putative link between the protein and soluble Aβ.

By investigating what could be the contribution of a conformational change of p53 in the pathogenesis of AD, we defined for the first time a hierarchical scale of events driven by Aβ.

In particular, in Part 1 we describe that Aβ induces the depletion of the homeodomain-interacting protein kinase-2 (HIPK2), an evolutionary conserved serine/threonine kinase whose activity is fundamental in maintaining wild-type p53 conformation and function, with subsequent abolishment of p53 transcriptional activity. We demonstrate that HIPK2 degradation driven by Aβ determines the loss of p53 transcriptional activity which, in turn, may contribute to AD pathogenesis leading to dysfunctional cells.

Starting from the discover of this novel amiloyd-based mechanism in AD, in Part 2 I highlight the potential relevance of HIPK2 as a new putative functional biomarker useful in addressing new therapeutic strategies against not only cancer but also important neurological conditions, such as AD.

After the list of papers published as result of my Doctoral work, the last part of this thesis is an extension of my interest in other scientific themes.

Chapter I

Unfolded p53: a new role as predictive biomarker of the progression from mild cognitive impairment to Alzheimer's disease

Introduction

p53 protein is a 53 KDa transcription factor constitutively expressed in most cells and tissues. The gene encoding p53 is located on human chromosome 17p13.1 which is one of the most common sites for genetic alterations in human solid cancer, since it is mutated in more than 50% cancer cases worldwide [1]. By the early 1990s, data from the first p53 knockout mice provided evidence of the potent tumor suppressor action of wild-type p53 [2]; subsequent research clearly demonstrated that the transcription factor is a key controller in cell cycle and determines the fate of the cell in response to oncogenic and to other stresses to maintain genomic integrity. Thanks to its role p53 has gained the name of "guardian of the genome" [3].

To achieve this goal p53 may use a wide spectrum of activities within numerous signalling pathways, such as its ability to function as transcription factor, by inducing or repressing different genes, as an enzyme, acting as exonuclease during DNA reparation and as a regulatory protein, among others. p53 oversees the correct implementation of processes and it intervenes only in case of

dangerous deviations from the proper cellular activity [4]. When the cell is exposed to critical conditions or undergoes damages p53 arbitrates cell faith [5] (Figure 1).

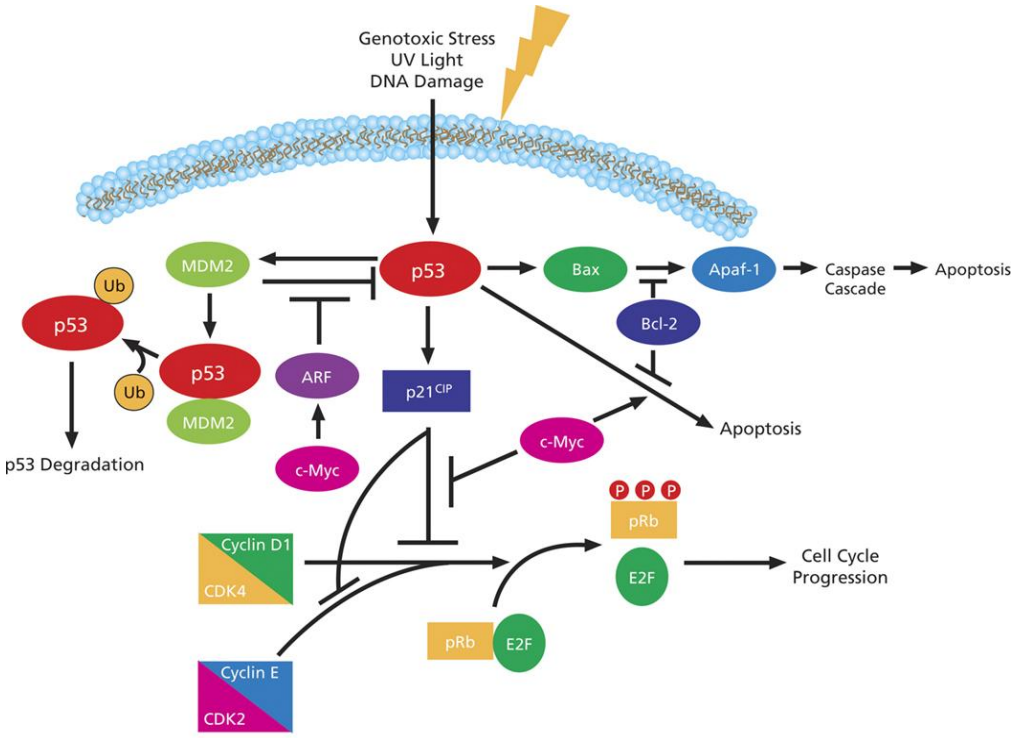

Figure 1: Exposure to multiple signals such as ionizing radiation, aberrant growth signals, chemotherapeutic drugs, ultraviolet light, hypoxia and oxidative stress induces p53 to accumulate in the nucleus to bind specific DNA target sequences and to activate or repress the transcription of genes participating in cell cycle control, in DNA repair and genes that induce cell death such as p21 and Bax, respectively. (Extracted from www.sigmaaldrich.com).

Rationale

Our group has studied p53 in fibroblasts from aged controls and demented patients finding that with aging there is an increase in the expression of an unfolded protein state, which is more pronounced in AD patients and is not dependent on gene mutations [6]. As a result of such conformational change, p53 partially loses its activity and shows a significant impairment in its DNA binding and transcriptional capacity when cells are exposed to a noxious stimulus [6]. In fact, AD fibroblasts are less vulnerable to oxidative injury than fibroblasts from non-AD subjects. Following these observations conformationally altered p53 has been proposed as putative biomarker for early AD [6]. This altered conformation can be experimentally recognized by an antibody (clone PAb240), which made these cells distinct from those of controls recognizing a primary epitope cryptic in wild-type p53 [7].

Recently, in cultured peripheral blood cells derived from AD patients we observed a detectable amount of unfolded p53, which made these cells distinct from those of controls and from patients affected by other form of dementia, suggesting that unfolded p53 could be used as a biomarker of the disease [8] (Figure 2).

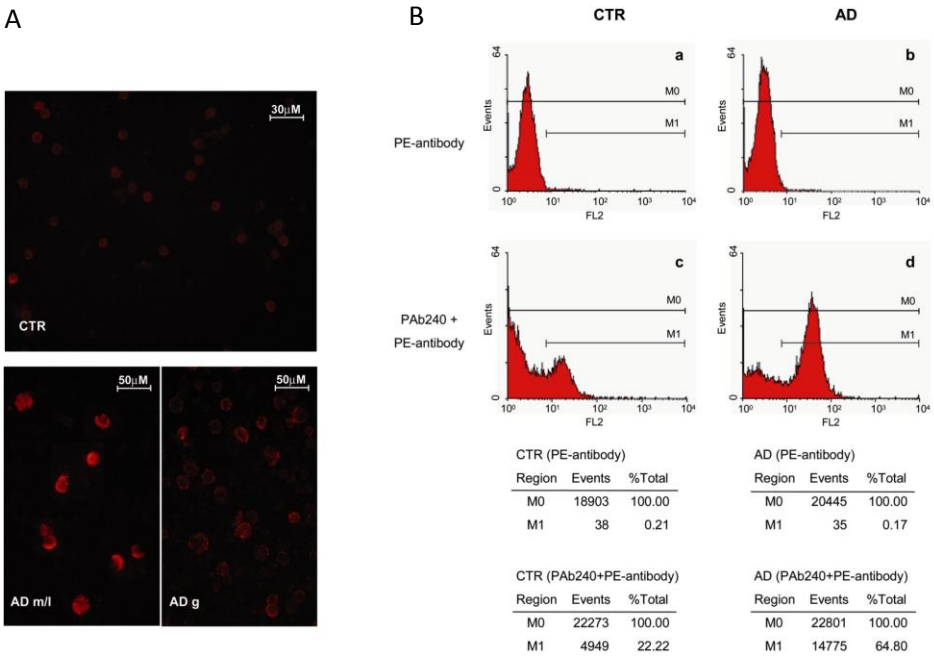

Figure 2: Immunofluorescence (A) and flow-cytometric analysis of blood cells from controls (CTR) and AD patients (B). (A) In the micrograph AD m/l, the fluorescence intensity of mononuclear cells (monocytes and lymphocytes, m/l) is evident as compared to the very faint background of CTR sample; figure ADg also showed some non-specific red fluorescence from polymorphonuclear cells (granulocytes, g). (B) Panels (a–d) are histograms reporting the fluorescence due to unfolded p53 expression (FL-2) vs. the number of reacting cells (events).

Before presenting p53 as marker of transition from MCI to AD, I will reflect on specific aspects of pharmacogenetics/pharmacogenomics of aging and age-associated diseases that involve p53.

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Part 1.

Unfolded p53 in the study of aging and age associated diseases from the perspective of pharmacogenetic/pharmacogenomics research

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PHARMACOGENETICS AND PHARMAGENOMICS, TRENDS IN NORMAL AND PATHOLOGICAL AGING STUDIES: FOCUS ON p53

Cristina Lanni, Marco Racchi, Daniela Uberti, Giuliano Mazzini, **Serena Stanga**, Elena Sinforiani, Maurizio Memo and Stefano Govoni

ABSTRACT

In spite of the fact that the aging organism is the result of complex life-long gene/environment interactions, making peculiar the susceptibility to diseases and the response to drugs, pharmacogenetics studies are largely neglected in the aged. Altered response to drugs, cardiovascular and metabolic alterations, cancer and dementia are among the age associated ailments. The latter two are the major contributors to illness burden for the aged. Aging, dementia and cancer share a critical set of altered cellular functions in the response to DNA damage, genotoxic stress, and other insults. Aging in higher animals may be influenced by the balance of cell survival versus death, a decision often governed by checkpoint proteins in dividing cells. The paper is mainly focused on one of such proteins, p53 which has been recently shown to be involved in aging and Alzheimer's disease (AD). Within this reference frame we studied p53 in aged controls and demented patients finding that with aging there is an increase of mutant like conformation state of p53 in peripheral blood cells, which is more pronounced in AD patients. As a result of such conformational change, p53 partially loses its activity and may become unable to properly activate an apoptotic program when cells are exposed to a noxious stimulus. Moreover we found that the tertiary structure of p53 and the sensitivity to p53-dependent apoptosis are affected by low concentrations of soluble beta amyloid, the peptide that accumulates in AD brain but also present in peripheral tissues. It is possible that p53 conformers may occur in the presence of misfolded molecules such as, but not limited to, beta

amyloid. In particular at neuronal level the altered function of cell cycle proteins may affect synaptic plasticity rather than cell duplication.

PHARMACOGENETICS AND PHARMACOGENOMICS: OPPORTUNITIES IN AGING STUDIES

The terms pharmacogenomics and pharmacogenetics tend to be used interchangeably, and a precise consensus definition of either remains elusive. However, pharmacogenetics refers to the role of genetic variation affecting drug response or adverse reactions to drugs [1]. While, pharmacogenomics better fits the definition of the science dealing with the analysis of genome (DNA) and of its products (RNA and proteins), aimed to correlate genomic information to cell or tissue response in order to find new targets for therapy, to develop new drugs and to study the response to them (see Drug Information Association website at www.diahome.org).

The aging organism is the result of complex life-long gene/environment interactions, making peculiar the susceptibility to diseases and the response to drugs. Nowadays, altered response to drugs, cardiovascular and metabolic alterations, cancer and dementia are among the age associated ailments. In this context, the shift from broad treatment strategies to more individually and genetically selected approaches would ensure that therapies would be both safer and more effective. In fact with an increased understanding about how genes and drugs interact, many patients could undergo a genetic test to predict their response and help ensure the medicine and dose is right at the first time. However, pharmacogenetic studies in aged patients are largely neglected, even if in elderly people pharmacokinetic and/or pharmacodynamic changes occur compared to younger people, increasing the variability of the response. Aging is

characterized by a progressive loss of functional capacities of most if not all organs, a reduction in homeostatic mechanisms and a response to receptor stimulation. Also, loss of water content and an increase of fat content in the body are reported. These changes have to be taken into consideration when the prescription of particular (cardiovascular or neuropsychiatric) drugs in elderly patients increases the risk of adverse drug reactions [2]. Pharmacodynamic changes in the elderly can result in greater, or sometimes even lesser, drug sensitivity than that seen in a younger individual. A greater drug sensitivity is particularly noticed with those drugs which act on the central nervous system, such as benzodiazepines [3]; on the other hand, β-adrenergic agents are an example of a reduction in responsiveness in elderly individuals [4]. Even if there is a general trend of greater pharmacodynamic sensitivity in the elderly, however, this is not universal, and these age-related changes must be investigated agent-by-agent. Thus, inter-individual differences in drug response and adverse effects may be caused by variability in drug metabolism due to genetic polymorphisms, induction or inhibition of the metabolism by concomitant drug intake, environmental or physiological factors or pathological conditions. As an example, the metabolism of donepezil, a selective acetylcholinesterase inhibitor used in the treatment of Alzheimer's disease (AD), is dependent upon the genetic polymorphisms of the gene encoding CYP2D6, which has a large number of allelic variants, causing either absent, decreased or increased enzyme activity [5-6]. Varsaldi and coworkers [7] observed a large inter-individual variability in the concentration to dose ratio of

donepezil among AD patients and noted that this variability partially correlated with the CYP2D6 genotype, thus suggesting that genetic polymorphism of CYP2D6 influences the metabolism and therapeutic outcome of donepezil.

Compared to pharmacogenetics, an abundant literature on pharmacogenomic studies is available even if not yet well organized. To date, pharmacogenomic studies are mainly focusing on many questions related to aging, concerning the research for aging genes, disease causing genes, longevity genes and are also pointing to identify susceptibility genes related to specific diseases, which allow health care providers to predict more accurately the risk of an individual of developing a specific disease [8-9]. A number of laboratories are making substantial and exciting progresses in the understanding of the genetics of aging and longevity; the principal aim of this studies are that these gene discoveries will lead to the identification of drug targets-drugs that would slow down the aging process and permit people to delay and perhaps escape age-associated diseases. Interestingly, by overviewing PubMed Citations from 1950 to 2008, the choice of model organism for aging is represented by *Homo sapiens*. In fact, while we found many data about developmental biology from worms, flies, and mice, there is a paucity of detailed information on the pathophysiology of aging, particularly in worms and flies; in contrast, there is a vast literature on these and all other aspects of human biology, including remarkable progress in human genetics [10]. Aging and susceptibility to diseases associated with aging are likely to be influenced by thousands of genes [11-12].

The vast amount of available literature generated in this field needs, however, a systematic revision and confirmation studies for the frequently contrasting results. Lacking genetic variations that predispose to diseases (so called “disease genes”) as well as having variations that confer disease resistance (so called “longevity-enabling genes”) are probably both important to confer a remarkable survival advantage [13-14]. Conversely, defective functions of genes associated with longevity may influence premature neuronal survival [8].

About aging genes, the debate regarding the existance of genetic mutations actually able to either cause or accelerate aging is still open. Some researchers have proposed a genetically set biological clock which establishes when a species begins to age. Telomere length and/or telomerase function have been selected as time keeper candidates because of their potential role in regulating the number of cell divisions [15]. However, reduction in telomerase activity with older age might actually be a defensive measure against the increased potential for age-associated cancer. Another genetic defense against cancer is the p53 gene. Tyner and colleagues have noted an increase in p53 activity as a result of a deletion mutation in the first six exons of the p53 gene associated with cancer prevention but also accelerated aging [16]. In fact, by generating transgenic mice carrying this mutation they found that mutant p53 mice exhibit an early onset of phenotypes associated with aging (reduced longevity, osteoporosis, generalized organ atrophy and a diminished stress tolerance), thus suggesting that p53 has a role in regulating organismal aging [16].

Among susceptibility genes, there are now numerous examples of genetic variations that substantially predispose to age-related disease (for example Alzheimer's disease and apolipoprotein E ε4). The apolipoprotein E (APOE) gene is the most prevalent risk factor for AD, especially in those subjects harboring the APOE-4 allele, whereas carriers of the APOE-2 allele might be protected against dementia [8, 17-18]. Because these variations are also associated with increased mortality risk, it is likely that centenarians do not have many of these predisposing variations [18-20]. Within this context, Schachter and colleagues found that the frequency of the apolipoprotein ε4 allele decreases markedly with advancing age, whereas, one of its counterparts, the ε2 allele, becomes more frequent with advanced age among Caucasians [18].

STRATEGIES FOR PHARMACOGENOMIC STUDIES

Cardiovascular and metabolic alterations, cancer and dementia represent complex age-associated diseases with multiple gene variations. Within the context of complex diseases, two different approaches are at the base of pharmacogenomic studies: one relies on a wide-genome screening and the other is driven by a specific hypothesis.

A genome-wide screening strategy underpins on the fact that, with the completion of the human genome project, single nucleotide polymorphisms (SNP) genotyping is being undertaken in a large number of pharmacogenomic studies to identify variants associated with responses to specific drugs. Throughout the human genome,

several types of genetic polymorphisms can be found, but among these, SNPs are the most common polymorphisms and the attention of researchers has been focused on these in the last years. Genome-wide association studies have emerged as an increasingly effective tool for identifying genetic contributions to complex diseases and represent the next frontier to further understand the underlying etiologic, biological, and pathologic mechanisms associated with chronic complex disorders. There have already been success stories for diseases such as diabetes mellitus [21]. Another recent example of wide-genome screening comes from the Framingham Heart Study (FHS) [22]. AD provides another good example of a complex age-associated disease with multiple genetic etiologies, including specific sub-types inherited as autosomal dominant traits, as well as common forms related to the inheritance of susceptibility genes. By performing genome-wide associations, an expectation is that the multiple contributing loci (as in AD) can be identified simultaneously, and then integrated into pathways contributing to a pathogenetic process for which drugs can be identified [23-24]. There is now experimental and clinical trial evidence to support this prediction [21, 25-27]. Roses and coworkers described the design of high throughput disease-association SNP studies in AD [28], by confirming, from this initial screen that, among others, APOE4 is genetically associated with AD. Another screening strategy based on the use of microarray technology was also adopted for a new molecular test for AD in blood plasma [29]. Despite the great impact and the variety of available information, the screening strategy in pharmacogenomic studies

shows to date limitations yet. Currently, costs limit the widespread use of pharmacogenomics. Execution of pharmacogenomic studies is dependent on many pieces of information: access to accurate clinical and demographic data, DNA samples from well designed studies, SNPs, genotyping technologies, informatics technologies to handle large quantities of data, statistical methodologies for data analysis and interpretation. In this regard, other limitations in the progress of pharmacogenomics include tools used for collecting, archiving, organizing and interpreting the huge amount of data generated in a pharmacogenomics study so that data from different experiments can be compared.

Specific hypothesis driven studies represent another approach in pharmacogenomics. Differently from widegenome screening, the strategy is focused on the knowledge of well-known genes involved in specific diseases. Taking as example AD, functional genomics studies in AD revealed that age of onset, brain atrophy, cognitive decline, apoptosis, immune function and amyloid deposition are associated with AD-related genes. The genetic defects identified in AD during the past 25 years are represented by mutations in the amyloid precursor protein (APP) gene, on chromosome 21, in the presenilin 1 (PS1) gene, on chromosome 14, and in the presenilin 2 (PS2) gene, on chromosome 1 [8, 30-31]. In addition, polymorphic variants of risk in more than 200 different genes can increase neuronal vulnerability to premature death [18], with APOE gene (19q13.2) as the most prevalent risk factor for AD. Thus, focusing the study on one of these genes is possible to understand its specific role

on specific events typical of AD. On the other hand, instead, all together, these genetic factors could interact in still unknown genetic networks leading to a cascade of events characterized by abnormal protein processing and misfolding with subsequent accumulation of abnormal proteins, excitotoxic reactions, oxidative stress, mitochondrial injury, synaptic loss and deficiencies in neurotransmitter function [8, 30, 32-33].

Pertaining to the context of aging, another example of hypothesis-driven study is based on checkpoint control by specific proteins. Aging in higher animals may be influenced by the balance of cell survival versus death, a decision often governed by checkpoint proteins in dividing cells. One of such proteins is p53; in mammals, p53 loss increases tumorigenesis, while specific gain-of-function alleles reduce tumor incidence but accelerate aging, suggesting a trade-off between tumor surveillance and stem cell maintenance [34].

p53: AGING AND CRITICAL DECISION ABOUT CELL FATE

The transcriptional network of p53-responsive genes produces proteins able to interact with a large number of other signal transduction pathways in the cell. p53 protein can trigger the onset either of reversible or permanent growth arrest [35-37] or of apoptosis [38-39]. However, the mechanisms involved in the decision between these cellular responses are not well understood. Cell type, the presence of growth factors or oncogenes, the intensity of the stress signal, and the cellular level of p53 have been cited as important factors in determining a specific p53-induced response [40-42].

Posttranslational modifications of the p53 gene have also been reported to influence the response observed [43]. For example, p53 phosphorylation by different kinases in response to stress can select for arrest or apoptosis, suggesting the involvement of modifiers upstream of the p53 protein [43-44].

Once a cell has been damaged and the DNA damage response and p53 are activated, a complex signaling network is engaged to result in a long-term cell fate decision. Rodier and coworkers reported an intriguing scheme showing four options adopted in normal mammalian cells [34]. Activation of cell cycle checkpoints by p53 leads to transient cell growth arrest [45]; p53 physically localizes to sites of DNA damage to promote repair [46] and simultaneously stimulates the transcription of direct effectors of the cell growth arrest (e.g. the cyclin-dependent kinase inhibitor p21) as well as effectors required for effcient DNA repair of complex lesions that require longer processing (e.g. GADD45) [47]. At this point, several potential cellular outcomes can occur, most of which are heavily influenced by the cell type as well as the severity of the DNA lesions: transient cell cycle arrest (when DNA damage is not severe), defective repair (resulting in mutation, such as chromosomal aberrations), cell death (apoptosis) or permanent cell cycle arrest (cellular senescence) [34]. Thus, p53 protects the genome by promoting the repair of potentially carcinogenic lesions in the DNA, thereby preventing mutations. In addition, p53 eliminates or arrests the proliferation of damaged or mutant cells by the processes of apoptosis and cellular senescence [48-49]. Loss of function in p53 is usually associated to many

common human cancers. Mutant p53 is almost always defective for sequence-specific DNA binding, and thus for transactivation of genes upregulated by the wild-type protein [50]. Interestingly, when p53 is mutated in non-dividing cells, such as neurons, a dysfunctions accumulation can occur; Yang and coworkers, in fact, demonstrated the existence of aberrant neurons in AD brain, by showing that neurodegeneration is correlated to neurons reentering a lethal cell cycle [51].

CONFORMATIONALLY ALTERED p53 IN AGED CONTROLS AND ALZHEIMER'S DISEASE PATIENTS

We have studied p53 in aged controls and demented patients finding that with aging there is an increase of conformationally altered p53, which is more pronounced in AD patients, to the point that it has been proposed as a putative biomarker in the early stages of the disease [52-53]. As a result of such conformational change, p53 partially loses its activity and may become unable to properly activate an apoptotic program when cells are exposed to a noxious stimulus [52]. In this study we described and demonstrated an abnormal response of AD fibroblasts to an acute oxidative injury; in particular, fibroblasts from AD patients were found to be less vulnerable to the oxidative injury induced by H2O2 in comparison with fibroblasts from non-AD subjects. Fibroblasts from sporadic AD patients represent an important starting point in the research for novel biomarkers because of their various abnormalities in metabolic and biochemical processes, which reflect some of the events in AD brain [54-55].

Furthermore, on the basis of immunoprecipitation studies with conformation-specific p53 antibodies (PAb1620 and PAb240), which discriminated folded vs unfolded p53 tertiary structure, we found that in fibroblasts from AD patients a significant amount of total p53 assumes an unfolded tertiary structure; such alteration can compromise p53 response to an acute injury elicited by an excess of free radical production. Mutant p53 found in AD fibroblasts has been demonstrated to be independent from gene mutations on the basis of sequence analysis of the p53 gene, thus suggesting that one of the peripheral events associated to the disease is responsible for generating a conformationally altered p53 isoform [52]. In the attempt of investigating on the mechanism of such alteration, we assessed the contribution of APP metabolic products to the change in p53 conformational state. We found that the exposure to nanomolar concentrations of betaamyloid (Aβ) 1-40 peptide induced the expression of an unfolded p53 protein isoform in fibroblasts derived from non-AD subjects [56]. These data suggest that the tertiary structure of p53 and the sensitivity to p53-dependent apoptosis are influenced by low concentrations of soluble Aβ. On this basis, we hypothesised that low amounts of soluble Aβ induce early pathological changes at cellular level that may precede the amyloidogenic cascade. One of these changes is the induction of a novel conformational state of p53. If low amounts of Aβ peptide, not resulting in cytotoxic effects, are responsible for p53 structure changes, it could be possible to consider the unfolded p53 both as an

agent partecipating to the early pathogenesis and as a specific marker of the early stage of AD.

We then investigated the altered p53 isoform in more accessible cells, such as peripheral blood cells [53] to determine the frequency and the extent of this defect in AD patients and to explore the possibility to develop an assay to validate conformationally altered p53 as a putative peripheral marker of the disease. The identification of new biological markers, to date, can be greatly useful both to improve diagnostic accuracy and/or to monitor the efficacy of putative therapies, since the confirmatory diagnosis of AD is possible only *post mortem*, based on recognition and quantification of senile plaques and neurofibrillary tangles. Furthermore, we set up a rapid and easy flow-cytometric approach to identify the different expression of conformationally altered p53 between AD and non-AD subjects. Differently from other data in literature regarding the development of biomarkers for AD or in CSF [57-58] or in blood [59-60], the main advantage of our study was the development of a biological sample preparation procedure as well as of an analytical method that could permit a routine analysis. If this method should be confirmed in a larger population and further validated, it could be useful in improving the knowledge regarding the clinical diagnosis of AD, because we could have the possibility to recognize AD and non-AD cases, by starting from small blood volumes thus using a less invasive technique [53]. Interestingly, we observed that the expression of conformationally altered p53, both in controls and AD, is an age dependent event, while it is independent from the length of illness and

from the MMSE score; this linear correlation with age would suggest that its significance would be different within specific age interval segments. In order to determine the diagnostic performance of conformationally altered p53 as an AD marker, we worked out sensitivity and specificity within different age intervals and found that these values were more significant in subjects up to 70 years of age (sensitivity of 90% to discriminate AD patients from non demented aged individuals at a specificity value of 77%) compared with the corresponding values for individuals older than 70 years [53]. Although the age stratification is admittedly a post-hoc analysis it serves to indicate that the putative marker proposed appears to be more important in the younger patients, also considering that the positivity to p53 conformational mutant induced a calculated O.R. of 29.2 for AD. The fact that the most significant differences are observed in the youngest patients indicate that the measurement of conformational mutant p53 may be usefully applied to detect AD at the early stages, perhaps applied to those patients falling in the ill defined category of mild cognitive impairment (MCI). Whether this expression of conformationally altered p53 will be suitable as an adjunctive diagnostic tool to predict the conversion from MCI to AD is under investigation.

In addition, in order to better characterize the nature of this different expression between AD and control subjects, we also evaluated whether the expression of conformational mutant p53 showed a correlation with the APOE status. We recruited a population of 75 patients with sporadic AD, 69 healthy age-matched controls (CTR)

(Table 1). Genomic DNA was extracted from peripheral leukocytes by proteinase K digestion and standard phenol/chlorophorm extraction procedure. The APOE gene polymorphisms (isoforms APOE ε2, APOE ε3, APOE ε4) were determined by Hha I restriction endonuclease digestion of PCR products, according to Hixson and Vernier [61]. After having determined APOE gene polimorphisms, we then divided this population in four groups, control subjects and AD patients with and without the ε4 allele, to establish if the presence of the ε4 allele might further affect the expression of mutant-like p53. The expression of mutant conformational p53 was independent from distribution of ε4 allele of APOE, thus highlighting that the presence of ε3/ε4 and ε4/ε4 genotypes was not linked to a higher expression of p53 conformational status (Fig. 1). AD patients without the ε4 allele showed a higher expression of mutant-like p53 compared to respective controls (percent of PAb positive cells, mean ± SD; control subjects without APOEε4: 22.8 ± 12.2; AD without APOEε4: 39.3 ± 15.0; AD without APOEε4 versus respective control $P < 0.0001$). The comparison of the APOEε4 positive cases did not show a statistically significant difference between AD and the respective controls possibly because of the small size of the control sample (8 patients). On the other hand, complexively the p53 may be a marker useful in the ε4 negative younger patients (< 70 years).

	AD	CTR
N (M;F)	75 (20;55)	69 (35;34)
mean age ± SD	79 ± 9	78 ± 10
L.O.I. (month)	53 ± 26	
MMSE	14 ± 6	28 ± 1.6

Table 1. Demographic and Clinical Variables 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.

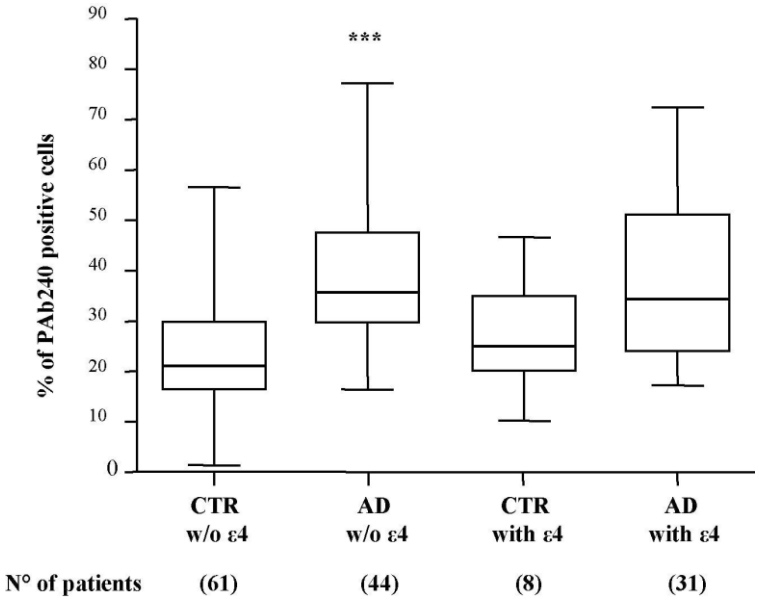

Figure 1: Correlation between the APOE status and conformational mutant p53. Box plot of the amount of conformational mutant p53 (%) expressed in

peripheral blood cells of all the subjects classified on the basis of their APOE genotypes. The figure is based on a rather innovative application in flow cytometry on peripheral blood cells. Venous blood samples were collected from a group of 75 patients with sporadic AD and 69 healthy age-matched controls (CTR). Peripheral blood mononuclear cells were then analyzed for the expression of conformationally altered p53 with a flow cytometer. The APOE gene polymorphisms (isoforms APOE ε2, APOE ε3, APOE ε4) were determined by Hha I restriction endonuclease digestion of PCR products. Bonferoni multiple comparison test was used to compare values in different groups. Differences were considered significant when a p-value < 0.05 was attained. ***p < 0.001 vs control without ε4 allele. We previously published [53] the observation that the elevated conformationally altered p53 was specific for AD and not observed in other neurodegenerative diseases, such as Parkinson's disease, vascular dementia and sopranuclear palsy.

CONCLUSIONS/PERSPECTIVES

Aging in higher animals may be influenced by the balance of cell survival versus death, at least in part regulated by a fine timing of checkpoint proteins and preservation of DNA integrity and correct repair [62-63]. With our study we propose an example pointing to a peculiar aspect of pharmacogenetic/pharmacogenomics research in aging and age associated diseases, that is the possibility to define novel targets for therapeutic interventions aimed to chekpoint proteins. p53 has been recently shown to be involved in aging and AD. Recent evidence suggests that increased p53 activity can, at least under some circumstances, promote organismal aging [34]. We showed a link between AD pathology and conformationally altered p53 [52-54], by finding that with aging a higher increase of an unfolded state of p53 in AD patients compared to age-matched

controls occurs. What can be the contribution of a conformational change of a protein to the aging process is under investigation. We could also address the issue whether a generalization of this phenomenon within the context of the “gain and loss of function” of protein conformers will be possible. It is worth to underline that the observation that aging and AD interfere with proteins controlling the duplication and cell cycle, such as p53, is interesting and may lead to the speculation that, in senescent neurons, derangements in proteins commonly dealing with cell cycle control and apoptosis could affect neuronal plasticity and functioning rather then cell duplication.

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Part 2.

From MCI to AD:
unfolded blood p53 as predictive biomarker

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UNFOLDED p53 IN BLOOD AS A PREDICTIVE SIGNATURE OF THE TRANSITION FROM MILD COGNITIVE IMPAIRMENT TO ALZHEIMER'S DISEASE

Cristina Lanni, Marco Racchi, **Serena Stanga**, Giuliano Mazzini, Alberto Ranzenigo, Renzo Polotti, Maurizio Memo, Stefano Govoni and Daniela Uberti

ABSTRACT

Mild cognitive impairment (MCI) is a syndrome defined as cognitive decline, but not sufficient to meet the criteria for any specific dementia. Although subjects with MCI may have an increased risk to develop AD, this clinical state encompasses several subtypes of cognitive dysfunction of different etiologies, none of which necessarily progresses to AD. The current inability of clinical criteria to accurately identify this at-risk group for AD development is fuelling the interest in biomarkers able to supplement clinical approaches. We recently described a blood-based cytofluorimetric method for conformationally altered p53 protein detection that allows the discrimination of AD patients from control subjects and patients affected by other dementias.

The same protein also predicted progression to AD in preclinical patients with MCI two years before clinical diagnosis of AD was made. Herein, we describe these findings and discuss the potential of the test in diagnosing AD.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by irreversible cognitive and physical deterioration. AD is the most common cause of dementia of the elderly population and its incidence doubles every five years between 65 and 85 years of age. It also represents a growing public health problem as life expectancy increases and the number of people with AD is expected to increase dramatically from approximately 24 million people in 2001 up to 81 million worldwide by 2040 [1]. The treatment of AD remains a major challenge because of an incomplete understanding of the events that lead to the selective neurodegeneration typical of AD brains. In view of existing and emerging therapeutic compounds, the focus has increasingly shifted to accurate detection of the earliest phase of illness.

The degenerative process in AD probably starts 20–30 years before the clinical onset of the disease [2]. This clinical phase, between prodromal and established AD, lies within the boundaries of the diagnosis of mild cognitive impairment (MCI), during which subjects have measurable cognitive deficits, but not sufficient to fulfill criteria for any specific dementia [3,4]. In particular, MCI subjects show the presence of subjective memory impairments, objective memory performance changes, and declining financial skills [5], whereas other cognitive functions and daily activities remain normal [6].

Although it is documented that subjects with MCI have an increased risk of AD, this clinical state encompasses several subtypes of cognitive dysfunction of varied etiologies, none of which necessarily

progresses to AD [7]. In particular, MCI subclassifications include an amnestic form (aMCI), characterized by isolated memory impairments, and one non-amnestic (naMCI) in which other cognitive functions rather than memory are mostly impaired [8]; within these groups, aMCI subjects showed 8.6-fold higher odds of developing AD [9]. Furthermore, it has been determined that patients suffering from aMCI progress to AD at a rate of 12 to 28% per year [3,10], whereas other MCI subjects improve, reverting to a normal level of cognitive functioning [11] and some die [12], thus underlining multiple etiologies for MCI. Hence, within this context, there is a need to reliably predict which patients with MCI will progress to AD. The current inability of clinical criteria to accurately identify this at-risk group is fuelling the emerging interest in biomarkers to potentially supplement clinical approaches.

Within this reference frame, we studied p53 in aged controls and demented patients finding that the conformational state of p53 protein in blood cells allows the differentiation of AD patients from control subjects and patients affected by other dementias. The notions about p53 come mainly from oncology. p53 has been described as a the guardian of the genome [13]. It is activated in response to certain stressful situations, inducing either cell cycle arrest and DNA repair or apoptosis [14]. The full-length p53 molecule comprises three major domains; the N terminus transactivation domain, the core DNA-binding domain, that is stabilized by a Zn2+ atom coordinated by a histidine and four cysteines, and the C terminus tetramerization domain. p53 is mutated in about half of all human cancers, with 95%

of these mutations occurring in the core domain [15]. In particular, the core domain is endowed with high flexibility, and certain missense mutations inside this central domain can affect tertiary structure of the protein.

Thermodynamic studies of p53 cancer mutations have identified three major phenotypes classified as: i) mutation affectingDNA contacts that have little impact on protein folding; ii) mutation disordering local structure leaving more than 85% of the protein in folded conformation; and iii) mutations globally denaturating the core domain, thus resulting in more than 50% of unfolded isoform [16–19]. This third phenotype is recognized by PAb240 antibody, since its epitope is cryptic when p53 is in wild type conformation and becomes accessible only when the core domain is denatured. It should be stressed that conformational changes of p53 can also be due to posttranscriptional modifications in the absence of specific mutations [20–23].

Within this context, the presence of an altered unfolded p53 isoform was found enhanced in AD patients in comparison with control and other types of dementia [24]. Notably, the best sensitivity and specificity of this marker was observed at ages 70 years [24], suggesting the idea that unfolded p53 could also be detected in the early stage of the disease.

On this basis, the purpose of our study was to further investigate whether conformationally altered p53 expression may be applied to those patients falling in the ill-defined category of MCI and in

particular to predict which subjects among MCI patients will progress to AD.

MATERIALS AND METHODS

Subjects

Venous blood samples from healthy people and from patients affected by MCI and AD were obtained from Sant'Orsola Hospital in Brescia, Italy. This population consisted in a group of 45 patients with sporadic AD, 45 healthy age-matched controls (CTR), and 28 patients with MCI (Table 1).

The protocol of the study, including the follow-up visits, was approved by the Ethical Committee and a written consentwas obtained fromall subjects or, where appropriate, their caregivers.

All the subjects were examined by a senior neurologist or geriatrician and diagnosis of dementiawas made according to DSM-IV and the NINCDS-ADRDA criteria. All MCI subjects met the original Petersen/Mayo criteria for MCI [3,7]. Dementia was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT scan) investigation. Cognitive statuswas quantified using the Mini Mental State Examination (MMSE). All AD patients fulfilled the criteria for probable AD [25] and were classified as "sporadic" on the basis that they lacked a familial history of the disease, as acquired from interviews with first degree relatives. Control subjects were aged individuals with no clinical signs of neurological or psychiatric diseases, mostly enrolled among spouses of the 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 interval clinical frame.

After two years from the recruitment, all 28 MCI subjects were recalled and examined by the geriatrician. The progression to AD was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT Scan) investigation.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll Hipaque density gradient from Na+/citrate samples (Eurobio, Italy) and fixed in 2% formaldehyde in 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 unfolded p53 (clone PAb240; NeoMarkers, Fremont, CA) (4 μg/ml in PBS/1% BSA solution). In particular the PAb240 antibody recognizes the epitope 211–217 aa inside the core domain, which is accessible only when the core domain is denatured and the protein assumes an unfolded tertiary structure, thus discriminating the so called “mutant-like” isoform [22]. Cells rinsed in PBS/1% BSA were incubated for 30 min in ice with a goat anti-mouse IgG antibody phycoerythrin (PE)-conjugated (DakoCytomation, Denmark; 1:40 in PBS/1% BSA). After rinsing, fewmicroliters of cell suspension were deposed on a glass slide and observed with a fluorescence microscope

Olympus BX51 with blue excitation (BP450–480 nm, DM 500, andbarrier filter 515 nm) equipped with a Camedia digital camera. Samples were observed at 40X magnification. Pictures were taken with the same instrumental setting. Orange-red fluorescence from AD positive cells was compared with the background fluorescence of control samples as a technical control of the experimental setting. The percentage of PAb240 positive cells was quantified by cytofluorimetric analysis. Cell suspension was analyzed with a flow cytometer Partec PASII (Partec, Germany). PBMC population was identified by forward and side angle scatter (FSC, SSC) and mutant p53 emission was detected in the FL-2 channel (535–580 band pass filter). For each sample, data from 20000 events were recorded in list mode, displayed on logarithmic scales and analyzed usingWinMDI 2.8 software.

	CTR	AD	MCI
N (M;F)	45 (20;25)	45 (9;36)	28 (5;23)
mean age ± SD	75 ± 12.6	81 ± 7.2	69 ± 12.4
L.O.I. (month)		52 ± 23	19 ± 6
MMSE	28.4 ± 1.6	14 ± 5.5	27.3 ± 2
Genotype	**CTR**	**AD**	**MCI**
ε2/ε3	5 (11)	2 (4)	3 (10.7)
ε3/ε3	35 (78)	25 (56)	17 (60.7)
ε3/ε4	5 (11)	14 (31)	7 (25)
ε2/ε4	0	0	1 (3.6)
ε4/ε4	0	4 (9)	0

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 (%).

Molecular genetic analysis

Genomic DNA was extracted from peripheral leukocytes by proteinase K digestion and standard phenol/ chlorophorm extraction procedure. The APOE gene polymorphisms were determined by Hha I restriction endonuclease digestion of PCR products, according to Hixson and Vernier [26].

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) followed when significant by an appropriate post hoc comparison test. Differences were considered significant when a p-value ≤ 0.05 was attained.

RESULTS

118 subjects were enrolled among those referring to the Department of Geriatric Medicine, S. Orsola Fatebenefratelli Hospital in Brescia, Italy. Control subjects and AD and MCI patients were comparable as far as age and gender distribution (see Table 1). The distribution of $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles of APOE was 0.022/0.733/0.244 in AD cases, and 0.056/0.889/0.056 in control subjects; the frequency of the APOE $\varepsilon 4$ allele was, as expected, higher in AD population than in control subjects. MCI showed a distribution of $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles of APOE of 0.071/0.786/0.143.

Blood mononuclear cells derived from AD and MCI patients and healthy subjects were subjected to cytofluorimetric analysis using the conformational specific antibody, PAb240, which discriminates

unfolded p53 tertiary structure. We only analyzed this population, since we previously demonstrated by immunofluorescence staining the presence of false positive cells, identified as granulocytes, showing under fluorescence microscope, a light diffuse mostly non specific signal, compared to the strong positivity to PAb240 antibody in monocytes and lymphocytes [24]. A highly statistically significant difference was found in the percentage of PAb240 positive cells when comparing controls with patients affected by MCI or AD subjects (percent of PAb positive cells, mean ± SD; control subjects: 23.4 ± 11.9; MCI: 30.6±10.7; AD: 35.1±11.3; MCI versus control $P<0.01$, AD versus control $P<0.001$) (Fig. 1).

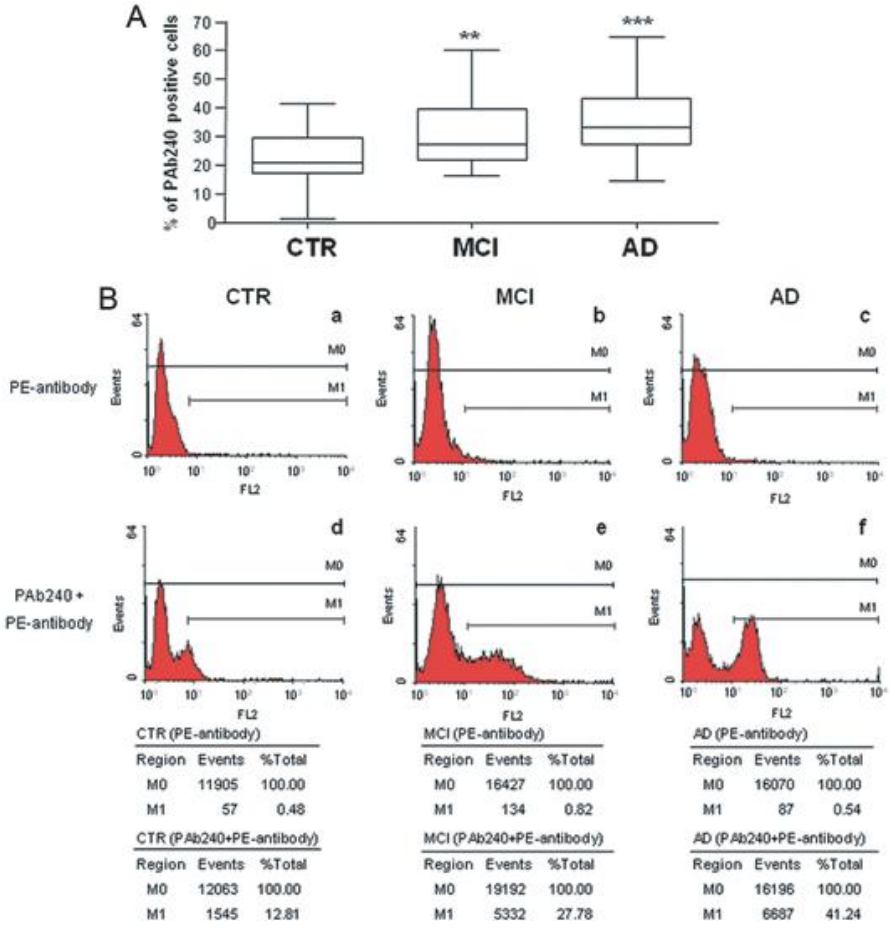

Figure 1: p53 protein expression in blood cells from AD, MCI and non-AD patients. (A) Box plot reporting the amount of conformationally altered p53 (%) expressed in peripheral blood cells for each subject enrolled in this study. Patients are divided according to the diagnostic group. Tukey-Kramer Multiple Comparisons Test has been used for statistical analysis. **$p<0.01$, ***$p<0.001$ vs control. (B) Quantitative analysis of conformationally altered p53 expression after flow-cytometric analysis. Panels a, b, c, d, e, f are histograms reporting the fluorescence due to conformationally altered p53 expression (FL-2) vs the number of reacting cells (events). For each histogram the correspondent statistical analysis is reported.

To better characterize the nature of this different expression among AD, MCI, and control subjects, we evaluated whether the expression of conformational mutant p53 showed a correlation with some parameters linked to AD. As previously reported [24], a statistically significant correlation was observed only when the expression of mutant conformational p53 and the age of both control subjects and AD patients were considered, thus confirming in another independent population that conformationally altered p53 is an age-dependent factor (data not shown).

Focusing on MCI group (28 patients), we found that 8 of them expressed APOEε4 allele. In addition, 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 35.3%), consistently with other previous data from literature [27].

The first group (n = 20) had percentages of unfolded p53 values ranging from 19.5% to 29.1% and the other group (n = 8) expressed values ranging 38.2% to 60.3%. The latter was candidate at high risk to develop AD, according to our previous indications [13]. Thus, according to the predicted criteria, 16 patients (8 with APOEε4 and 8 with high unfolded p53) were at high risk to develop AD.

Interestingly, the follow-up of MCI patients at two years from recruitment revealed that 14 out of 28 MCI patients converted to AD (Fig. 2). Among these, six had high values of unfolded p53, three expressed APOEε4 variant, and one had both high unfolded p53 and APOEε4. Four of the novel AD diagnoses were not predicted either

by the APOE genotype or by p53 conformational state (Fig. 2A). Among the nonconverted group (14 patients), four expressed the ε4 variant of APOE gene and only one had high unfolded p53 (Fig. 2B). Moreover, in the MCI converted group, the expression of conformational altered p53 was independent from distribution of ε4 allele of APOE (Fig. 2).

This is also consistent with our previous work [28], demonstrating that the presence of the ε4 allele of APOE does not influence the level of conformational mutant p53 expression. The combination of APOEε4 allele and unfolded p53 yielded a sensitivity of 71.4% and a specificity of 84%, comparable with data reported by Zetterberg et al. [29] on cerebrospinal fluid (CSF) biomarkers.

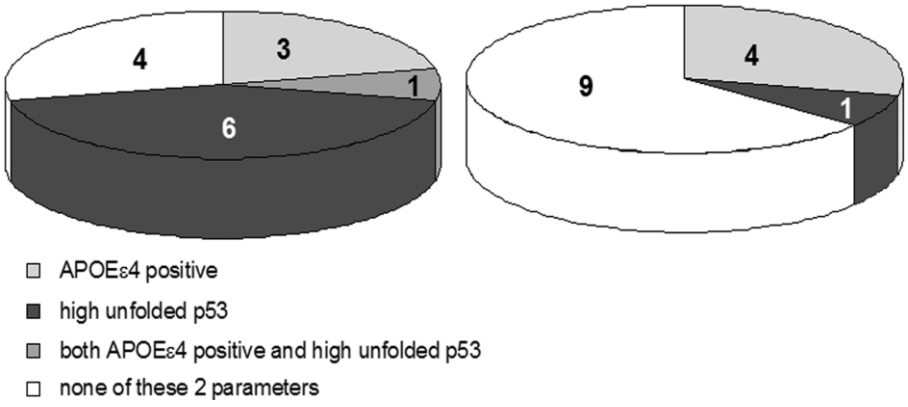

Figure 2: Scheme of converted and not-converted MCI patients based on unfolded p53 analysis and APOEε4 genotype. Twenty-eight MCI (5 males and 23 females) with a mean ± standard deviation age of 69 ± 12.4 and MMSE score of

27.3 $\pm$ 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 three groups according to APOEε4 variant and high unfolded p53. Two years later, patients were re-evaluated and classified as converted and non-converted in AD (A, B).

DISCUSSION

Recent research suggested that onset of AD is commonly preceded by an interim phase known as MCI. Since MCI diagnosis may include patients in a transitional state between prodromal and full blown AD, it is considered a window of therapeutic opportunity for treating patients prior to symptom onset [3], even if the presently available treatments are mostly symptomatic.

On the other hand, important new pharmacotherapeutic options will likely become available only over the next decade, considering the variety of drug targets and mechanisms of action identified and the total number of compounds under investigation [30]. In view of existing and emerging therapeutic compounds, there is increasing interest to develop techniques allowing an accurate detection of the earliest phase of the disease. The introduction of biological markers in the clinical management of AD will not only improve diagnosis relating to early detection of neuropathology with underlying molecular mechanisms but also provide tools for the assessment of objective treatment benefits.

Measurement of p53 conformational status in blood cells has been found to discriminate AD cases from normal aging, Parkinson's

disease, and other dementias [24]. 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 [24]. Since p53 conformational changes found in AD cells have been demonstrated to be independent of gene mutations [31], it is reasonable to wonder about the mechanism by which p53 changes its conformational state. This issue is actually under investigation in our laboratory. On the other hand, a pathogenic link between p53 conformational changes and AD was indirectly suggested by the effects of low soluble concentrations of amyloid-β (Aβ) on p53 tertiary structure in fibroblasts [32] and other cell lines [33]. It is noteworthy that Aβ and prooxidant conditions may induce conformational changes in p53. The effect itself of Aβ on p53 misfolding can be counteracted by vitamin E [33]. Indeed, pro-oxidant conditions in MCI and AD patients [34] may have driven the observed changes in PBMC. In addition the possibility that the p53 misfolding in MCI and AD patients reflects an overall propensity to protein misfolding cannot be ruled out.

In this study, to assess whether p53 conformational changes are an early event in AD pathology, we followed up patients falling in the MCI category for two years. Analysis of unfolded p53 was performed at the beginning of the enrollment and was used to predict which subjects among MCI patients will progress to AD. The results obtained in the present study confirm and extend the previous observations on AD patients. Furthermore,we found that a cytofluorimetric approach for conformationally altered p53 protein

was able to predict progression to AD in a small cohort of preclinical patients with MCI two years before clinical diagnosis for AD was made. The rate of progression of MCI to AD was comparable with the mean reported in population studies [3,10]. In fact we found that 50% of MCI patients converted to AD after two years from the recruitment. The high expression of unfolded p53 may be considered as high risk factor for the conversion to AD (among 8 patients with elevated unfolded p53 seven converted). In fact, in the MCI converted group, 50% was predicted based on elevated levels of conformationally altered p53, whereas only 28% was predicted based on APOE status. Noteworthy, higher levels of mutant conformational p53 and APOEε4 appeared to be independent risk factors for AD even if numbers are small to be strong at this point. In line with our previous data [28], complexively the mutant conformational p53 may be considered as a marker useful in the ε4 negative younger patients ($\leq$ 70 years).

Interestingly enough, when we combined the data on p53 and APOEε4, we obtained a calculated sensitivity of 71.4% and a specificity of 84% for the predictive test. Intriguingly, this accuracy was comparable with data reported by Zetterberg et al. [29] on combined CSF biomarkers.

The fact that p53 was able to identify only a subset of converting patients is consistent with the idea that single biomarkers may not have sufficient sensitivity to detect all cases of the disease or adequate specificity to distinguish one pathology fingerprint from other disease profiles. For this reason is important to identify a battery of

determinations to increase the accuracy of the diagnosis. In this context, several studies have showed that selected combinations of preclinical markers may predict this conversion [35–37]. The combined use of cognitive tests, APOE genotype and a neuroradiological technique has been proposed as the best option for prediction, showing an accuracy > 90% [38]. Other research groups focused on CSF, total-tau/Aβ1−42, and magnetic resonance imaging (MRI) biomarkers, such as hippocampal, entorhinal, and ventricular volumes and brain atrophy rates, finding that their combination provides better prediction than either source of data alone [39,40], with a diagnostic accuracy of 80–90%. However, it is difficult to prepare a patient for all these analyses and sequential studies appear difficult to perform, making routine testing not practical in elderly at-risk patients. This emphasizes the need for simple not invasive tests, useful to provide biomarkers that can identify MCI patients declining to AD. 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 [41]; furthermore these 18 proteins showed a potential in identifying patients with MCI who progress to AD [42]. However, the proteomic approach lacks of the identification of posttranscriptional protein modifications deriving from biochemical and metabolic events linked to the development of the pathology.

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 and, when associated with APOEε4 genotype, it yielded sensitivity and specificity values comparable to those obtained by a combination of 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 for MCI to AD requires a further investigation in larger and independent populations of patients, which is at the moment under investigation. Anyway, we suggest that measurement of conformational p53 state can be useful as an easy accessible adjunctive diagnostic tool in identifying those in the at-risk group of MCI patients who progress to AD.

Finally, it is important to underline that we cannot speculate at this time whether conformational altered p53 found in AD peripheral cells is also present in the brain of AD patients and the relevance of such impairment in terms of neuronal function. Studies in this direction are now in progress in our laboratory. There are, however, previous postmortem studies suggesting an involvement of p53 in degenerating neurons in AD. These include de la Monte and colleagues [43] showing increased p53 and Fas expression in specific populations of cortical neurons; Kitamura et al. [44] showing increased amount of p53 in temporal cortex, mainly localized in glial cells; and Seidl et al. [45] showing higher levels of p53 in frontal and temporal lobe from Down syndrome patients. On the other hand it is not known whether the increase in p53 observed in the quoted papers occurs in degenerating neurons and/or reflects the expression of a

conformational altered isoform of p53, as we detected in blood cells and fibroblasts from AD patients [24,31,32].

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Part 3.

Mild cognitive impairment conversion to Alzheimer's disease: advances on unfolded p53

In preparation as

MILD COGNITIVE IMPAIRMENT CONVERSION TO ALZHEIMER'S DISEASE: A PREDICTION STUDY WITH UNFOLDED p53

Serena Stanga, Cristina Lanni, Elena Sinforiani, Giuliano Mazzini, Stefano Govoni and Marco Racchi

Introduction

Mild cognitive impairment (MCI) is a transitional state between normal cognitive functioning and dementia. The current clinical criteria are unable to accurately identify the risk group for the progression to Alzheimer's disease (AD). The interest in biomarkers able to support clinical approaches is growing. We recently demonstrated that the detection of unfolded p53 allows to predict progression to AD in preclinical patients with MCI two years before the clinical diagnosis of dementia was evident [1].

In this study we determined to confirm the prognostic ability of unfolded p53 as predictive signature from MCI to AD in a longitudinal study on an independent population of patients.

We examined the expression of unfolded p53 in patients up to 70 years with MCI in order to discriminate this population from aged-matched control subjects and AD patients. MCI patients have been evaluated every six months for at least two visits from the beginning of the enrollment comparing the measurement of unfolded p53 with data from neuropsychological evaluation, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT Scan and RMI) investigation.

Preliminary data

Sixty-seven subjects were enrolled among those referring to the Institute 'Fondazione Casimiro Mondino' in Pavia, Italy. In this study we analyzed data from a group of 24 control subjects, 28 MCI and 15 AD patients comparable as far as age and gender distribution (see Table 1).

	CTR	MCI	AD
N (M;F)	24 (12;12)	28 (11;17)	15 (6;9)
age	71 ± 6	70 ± 7	75 ± 5
L.O.I. (months)		28 ± 13	34 ± 11
MMSE	29 ± 1	26 ± 2	21 ± 2
Temporal atrophy (Yes;No)	24 (0;24)	28 (4;24)	15 (11; 4)
Genotype	CTR	MCI	AD
ε2/ε3	2 (8)	1 (4)	2 (13)
ε3/ε3	22 (92)	21 (75)	6 (40)
ε3/ε4	0	4 (14)	6 (40)
ε2/ε4	0	0	1 (7)
ε4/ε4	0	2 (7)	0

Table 1: Demographic and clinical variables and genotype frequency of the APO-E alleles of all the subjects enrolled in the study. 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 (%).

The protocol of 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-ADRDA criteria. All MCI subjects met the original Petersen/Mayo criteria for MCI [2, 3]. Dementia was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT Scan and RMI) 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. Within each cognitive domain several aspects of function were assessed in order to obtain as complete picture as possible of the cognitive status of the subjects. 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.

On the basis of our previous results [1, 4], blood mononuclear cells derived from healthy subjects, MCI and AD patients were subjected to cytofluorimetric analysis using the conformational specific antibody, PAb240, which discriminates unfolded p53 tertiary structure. As previously reported, a statistically significant difference was found in the percentage of PAb240 positive cells when

comparing controls with patients affected by MCI or AD (percentage of PAb240 positive cells, mean ± standard deviation (SD); 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 $P < 0.01$).

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 [5].

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 40% to 63.3%. The latter group, according to our original hypothesis, was candidate at high risk to develop AD. After a mean of 15 months from the recruitment, 7 out of 28 MCI patients converted to AD. Among these, four expressed APOEε4 variant, four presented MT atrophy on MRI and all of them were predicted by high levels of unfolded p53.

Only a subset (7/13) of p53 identified at risk patients converted to AD. This is consistent with the idea that a single biomarker, such as unfolded p53, may not be sufficient to detect invariably converters versus non converters. For this reason is important to include a series of other biomarkers (i.e. the APOE gene polymorphisms, neuroimaging, neuropsychological battery of tests) to increase the accuracy of the diagnosis.

However, we cannot rule out that probably the 6 MCI patients at high risk of conversion, which expressed elevated levels of unfolded p53,

will convert at two years follow-up according to the rate of progression to dementia documented in literature [2, 3].

These results confirmed that the high expression of unfolded p53 may be considered as high risk factor for the conversion to AD and that it is important to identify a battery of determinations.

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Chapter II

Pathogenetic link between unfolded p53 and Alzheimer's disease

Introduction

Being the center of a complex organism regulation p53 serves different activities to ensure genomic integrity in cells.

p53 acts as enzyme, operating as exonuclease during DNA repair, as a regulatory protein in numerous signaling pathways and as transcription factor, which is the most studied and functionally significant activity of the protein [1]. In fact, p53 has a domain composition typical for transcription factor able to bind specific DNA sequences and to transactivate a number of genes, by inducing or repressing them [2].

p53 transcriptional activity depends mainly on post-translational modifications and protein/protein interactions that comprise a large network of covalent changes inducing characteristic modifications within the protein quantity, activity and ability to interact or cooperate with a variety of other proteins [3].

Another important mechanism that controls p53 transcriptional function is the conformational stability since p53 is an intrinsically unstable protein.

Rationale

Our research group has demonstrated, both in fibroblasts and in blood mononuclear cells from aged controls and demented patients, that there is with aging an increase in the expression of an unfolded p53 protein state which is more pronounced in AD patients and is not dependent on gene mutations [4, 5]. As a result of such conformational change, p53 partially loses its activity and shows a significant impairment in its DNA binding and transcriptional capacity when cells are exposed to a noxious stimulus [4]. This altered conformation can be due to a loss of zinc (Zn^{2+}) ion in the core domain of the protein, that provides the basic scaffold for the DNA binding and which has been demonstrated to be crucial for the stabilization of p53 in the so called "wild-type" folded form, recognized by the antibody PAb1620. Exposure of wild-type p53 to metal chelators such as ethylenediaminetetraacetic acid (EDTA) or orthophenanthroline determines a rapid switch to the unfolded form positive to the antibody PAb240 [6]. Upon addiction of micromolar amounts of Zn^{2+}, the protein undergoes a refolding to the native form and reacquires DNA-binding competence [6] (Figure 1).

Trying to investigate the cause of this alteration we found that the exposure to nanomolar concentrations of the beta-amyloid peptide 1-40 (Aβ 1-40) induced the expression of the unfolded p53 protein isoform in fibroblasts derived from non-AD subjects [7]. These data suggest that the tertiary structure of p53 and the sensitivity to p53-dependent apoptosis is influenced by low concentrations of soluble Aβ.

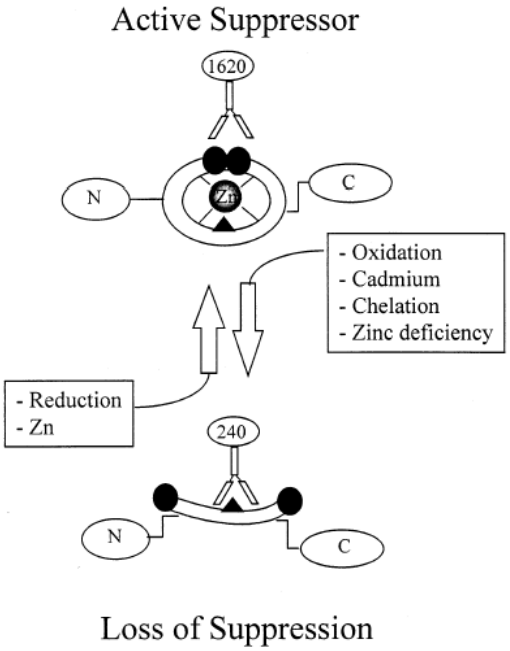

Figure 1: Micromolar amounts of Zn^{2+} determines the protein refolding to the native wild-type conformation and the DNA-binding competence.

On this basis, we assumed that low amounts of soluble Aβ induce early pathological changes at cellular level that may precede the amyloidogenic cascade and one of these changes is the induction of the unfolded state of p53, suggesting a role of the protein in the early pathogenesis of AD [7].

Recent evidence shows that the homeodomain interacting protein kinase 2 (HIPK2) is an evolutionary conserved serine/threonine kinase whose activity is fundamental in maintaining wild-type p53 function, thereby controlling the destiny of cells when exposed to DNA damaging agents [8]. Hence, conditions that induce HIPK2

deregulation may result in a dysfunctional response to stressors by affecting p53 activity.

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Part 1.

A novel amyloid-based pathogenetic mechanism in Alzheimer's disease involving HIPK2 and unfolded p53

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HOMEODOMAIN INTERACTING PROTEIN KINASE 2: A NOVEL TARGET FOR ALZHEIMER'S BETA AMYLOID LEADING TO MISFOLDED p53 AND INAPPROPRIATE CELL SURVIVAL

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ABSTRACT

Homeodomain interacting protein kinase 2 (HIPK2) is an evolutionary conserved serine/threonine kinase whose activity is fundamental in maintaining wild-type p53 function, thereby controlling the destiny of cells when exposed to DNA damaging agents. We recently reported an altered conformational state of p53 in tissues from patients with Alzheimer's Disease (AD) that led to an impaired and dysfunctional response to stressors.

Here we examined the molecular mechanisms underlying the impairment of p53 activity in two cellular models, HEK-293 cells overexpressing the amyloid precursor protein and fibroblasts from AD patients, starting from recent findings showing that p53 conformation may be regulated by HIPK2. We demonstrated that beta-amyloid 1–40 induces HIPK2 degradation and alters HIPK2 binding activity to DNA, in turn regulating the p53 conformational state and vulnerability to a noxious stimulus. Expression of HIPK2 was analysed by western blot experiments, whereas HIPK2 DNA binding was examined by chromatin immunoprecipitation analysis. In particular, we evaluated the recruitment of HIPK2 onto some target promoters, including hypoxia inducible factor-1α and metallothionein 2A.

These results support the existence of a novel amyloid-based pathogenetic mechanism in AD potentially leading to the survival of injured dysfunctional cells.

INTRODUCTION

The protein p53 responds to a variety of cellular stresses and may induce cell cycle arrest or apoptosis In fact, by differential activation of a large number of target genes and by mitochondrial functions, p53 is able to sense the intensity of the damage and modulate biological responses that can range from transient growth arrest to permanent replicative senescence or apoptosis [1].

The induction of p53 transcriptional activity depends mainly on posttranslational modifications together with protein/protein interaction [2]. Another important mechanism that controls p53 function is its conformational stability since p53 is an intrinsically unstable protein whose structure includes one zinc atom as an important co-factor for DNA-binding activity in vitro and in vivo [3,4]. An increased content of an unfolded p53 protein isoform [5–7] has been reported in numerous tumour cells where p53 harboured different gene point mutations. On the other hand, conformational changes of p53 towards unfolded isoforms are not only associated with gene mutations, but post transcriptional modifications can affect p53 tertiary structure. It is worth noting that in cellular models of Alzheimer's Disease (AD), p53 was found to be conformationally altered, making these cells less vulnerable to stressors or genotoxic insults [5–7]. When investigating the mechanism of this alteration, we found that the exposure to nanomolar concentrations of beta-amyloid (Aβ) 1-40 peptide was responsible for the increased content of unfolded p53 protein [8]. One of the activators that induces p53 posttranslational modification and wild-type conformational stability

is homeodomain interacting protein kinase 2 (HIPK2), an evolutionary conserved serine/threonine kinase able to regulate gene expression by the phosphorylation of transcription factors and accessory components of the transcription machinery. HIPK2 is activated in response to DNA damaging agents or morphogenic signals, thus playing a key role in differentiation, development or apoptosis [for a review see refs 9 and 10]. HIPK2 interacts physically and functionally with p53 and specifically phosphorylates p53 at serine 46 (Ser46) in response to severe DNA damage, regulating p53-induced apoptosis [11,12]. In addition, it has recently been shown that HIPK2 depletion results in p53 protein misfolding, changing the wild-type conformation to a conformationally altered status with subsequent abolishment of wild type p53 DNA binding and transcriptional activity. This can be restored with zinc supplementation [13].

Our purpose was to evaluate whether the altered conformational state of p53, observed in AD cells, was dependent on an impaired HIPK2 function and, by the use of two cellular models of AD, to define whether a deregulation of HIPK2 is involved in AD pathogenesis. Furthermore, since AD is characterized by an aberrant metabolism of the amyloid precursor protein (APP), in turn resulting in an aberrant production of Aβ peptides [14], our intent was to investigate the effect of Aβ peptides on HIPK2 expression and DNA-binding activity. The results presented here may define a hierarchical scale of events related to Aβ activities and eventually lead to a better understanding of AD pathogenesis.

MATERIALS AND METHODS

Reagents and cell treatments

All culture media, supplements and Foetal Bovine Serum (FBS) were obtained from Euroclone (Life Science Division, Milan, Italy). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA, USA). All other reagents were of the highest grade available and were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Amyloid-β (Aβ) peptide 1-40 and Ab 40-1 reverse peptide were solubilised in DMSO at the concentration of 100 mM and frozen in stock aliquots that were diluted at the final concentration of 10 nM prior to use. For each experimental setting, one aliquot of the stock was thawed out and diluted at the final concentration of 10 nM to minimize peptide damage due to repeated freeze and thaw. The Aβ concentration was chosen following dose response experiments (data not shown) where maximal modulation of p53 structure and its transcriptional activity [5] was obtained at 10 nM. All the experiments performed with Aβ were made in 1% of serum. Doxorubicin was solubilised in H2O at the concentration of 10 mM and frozen in stock aliquots that were diluted to working concentration (3.4 mmol/L) in medium at the moment of use. Zinc Chloride (ZnCl2) was diluted into the cell medium at 100 mM concentration for 16 h. To test proteasome activity, 6 hours before the end of treatment MG132 (Calbiochem, San Diego, CA, USA) was added to the medium at the concentration of 10 mmol/L.

Cell cultures

Skin Fibroblasts from two non-AD controls (2 females, mean age 63.569.2 years) and two AD patients (1 female, 1 male, mean age 66.0611.3 years) were selected from the cell lines present in our cell repository originally established in 1993 [33]. The diagnosis of probable AD was made according to the criteria developed by National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA). All AD patients presented a 1–4 year history of progressive cognitive impairment predominantly affecting memory. Non-AD patients were without established cognitive disorders. Neither AD nor non-AD patients presented neoplastic diseases at the time of tissue biopsy. All cell lines were frozen at passage 2–4 in a modified growth medium containing 20% foetal bovine serum and 10% dimethylsulfoxide. For the experiments, cell lines were simultaneously thawed and grown up to passages 7–10. Cells were cultured as previously described [21]. Each set of experiments was done using cells at the same passage (ranging from 7 to 10), carefully matching AD and non-AD samples. Culture conditions were kept constant throughout the experiments. Human embryonic kidney (HEK) 293 cells from European Collection of Cell Cultures (ECACC No. 85120602) were cultured in Eagle's minimum essential medium containing 10% foetal bovine serum, glutamine (2 mM), penicillin/streptomycin (2 mM), at 37uC in 5%CO2/95% air [5]. The HEK-293 cells stably transfected with APP751 were

obtained as previously described [5] and maintained in G418 at a final concentration of 400 mg/ml.

Transfection, plasmids and Western immunoblotting

Transient transfection was carried out using the N,N-bis-2-hydroxyethyl)-2-amino-ethanesulphonic acid-buffered saline (BBS) version of the calcium phosphate procedure and the following plasmids were used: HIPK2-Flag [11] and HIPK2-K1182R (MDM2-resistant) mutant [34]. Total cell extracts were prepared as previously described [20] and immunoblotting was performed with mouse monoclonal anti-Flag (Sigma Chemical Co., St. Louis, MO, USA) and mouse monoclonal anti-tubulin (Immunological Sciences, Rome, Italy). Immunoreactivity was detected by enhanced chemiluminescence kit (Amersham, Little Chalfont, UK).

p53 conformational immunoprecipitation

p53 conformational state was analyzed by immunoprecipitation as detailed previously [7]. Briefly, cells were lysed in immunoprecipitation buffer (10 mM Tris, pH 7.6; 140 mM NaCl; and 0.5% NP40 including protease inhibitors); 100 mg of total cell extracts were used for immunoprecipitation experiments performed in a volume of 500 ml with 1 mg of the conformation-specific antibodies PAb1620 (wild-type specific) or PAb240 (mutant specific) (Neomarkers, CA, USA). Immunocomplexes were separated by 10% SDS-PAGE and immunoblotting was performed with rabbit antip53 antibody (FL393) (Santa Cruz, CA, USA). Immunoreactivity was

detected with the ECL-chemiluminescence reaction kit (Amersham, Little Chalfont, UK).

RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The first strand cDNA was synthesized by reverse-transcribing 5 mg of mRNA with Moloney murine leukaemia virus reverse transcriptase kit and random primers (Applied Biosystems, Foster City, CA, USA). Semiquantitative RT-PCR was carried out by using Hot-Master Taq (Eppendorf, Milan, Italy) with 2 ml cDNA reaction and genes specific oligonucleotides under conditions of linear amplification. DNA products were run on 2% agarose gel and visualized by ethidium bromide staining using UV light. Data presented are representative of at least three independent experiments.

Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitation (ChIP) analysis was carried out essentially as described [13]. Protein complexes were crosslinked to DNA in living cells by adding formaldehyde directly to the cell culture medium at 1% final concentration. Chromatin extracts containing DNA fragments with an average size of 500 bp were incubated overnight at 4°C with milk shaking using polyclonal anti-HIPK2 antibody (Santa Cruz, CA, USA). DNAprotein complexes were recovered with protein G Agarose (Pierce, Rockford, IL, USA). Before use, protein G was blocked with 1 mg/ml sheared herring

sperm DNA and 1 mg/ml bovine serum albumin (BSA) overnight at 4°C and then incubated with chromatin and antibodies for 3 hrs at 4°C. PCR was performed using immunoprecipitated DNA and specific primers for human HIF1a and MT2A promoters [15,17]. Immunoprecipitation with non-specific immunoglobulins (No Ab) was performed as negative controls. PCR products were run on a 2% agarose gel and visualized with ethidium bromide staining using UV light. The amount of precipitated chromatin measured in each PCR was normalized with the amount of chromatin present in the input of each immunoprecipitation.

Transactivation assay

For transactivation assay HEK-293 and HEK-APP cells were transfected with the p53-target promoter AIP1-luciferase reporter plasmid (kindly provided by H. Arakawa, National Cancer Center, Tokyo, Japan), MT2A-luc (kindly provided by Jean-Mark Vanacker, UMR5242 CNRS/INRA/Universite´ Claude Bernard Lyon/ENS, Lyon, France) or HIF-1α-p800-luc (kindly provided by Carine Michiels, Laboratory of Biochemistry and Cellular Biology, FUNDP-University of Namur, Belgium) reporter plasmids, by using the cationic polymer transfection reagent jetPEI (PolyPlus-transfection, Illkirch, France) according to the manufacturer's instructions. Twenty-four hours later the cells were incubated with 100 mM Zinc Chloride (ZnCl2) for 16 h and with 3.4 mM doxorubicin for 24 h before luciferase activity was assayed. Transfection efficiency was normalized with the use of a co-transfected pCMV β-galactosidase

plasmid (β-gal). Luciferase activity was assayed on whole cell extract and the luciferase values were normalized to β-gal activity and protein content. At least three independent experiments were performed in duplicate.

Densitometry and statistics

All the experiments, unless specified, were performed at least three times.

Following acquisition of the Western blot image through an AGFA scanner and analysis by means of the Image 1.47 program (Wayne Rasband, NIH, Research Services Branch, NIMH, Bethesda, MD, USA), the relative densities of the bands were analyzed as described previously [35]. The data were analyzed by analysis of variance (ANOVA) followed when significant by an appropriate post hoc comparison test as indicated in figure legend. The reported data are expressed as means 6 SD of at least three independent experiments. A p value < 0.05 was considered statistically significant.

RESULTS

Aβ is responsible for HIPK2 deregulation

We first investigated whether HIPK2 DNA-binding to target promoters was somehow compromised by nanomolar concentrations of soluble Aβ peptides. To this aim, we treated HEK-293 cells with soluble Aβ 1-40 at the concentration of 10 nM for 48 h. As we have previously reported, this enters the cells and induces conformational changes in p53 protein [5]. Subsequently chromatin

immunoprecipitation (ChIP) experiments were performed in order to evaluate the integrity of interaction between HIPK2 and the hypoxia inducible factor-1a (HIF-1α) promoter, as recently reported [15]. As shown in Figure 1a, HIPK2 binding to HIF-1α promoter was eliminated by Aβ 1-40 treatment, whereas treatment with the reverse peptide Aβ 40-1 failed to do so. We further evaluated Aβ 1-40-induced HIPK2 downregulation and mRNA expression was assessed in HEK-293 cells after treatment with Aβ 1-40. As shown in Figure 1b, the analysis of RT-PCR following normalization to GAPDH expression revealed that no differences in HIPK2 mRNA expression were observed when comparing treated to untreated HEK-293 cells. Next, HIPK2 protein expression was evaluated by Western immunoblotting. As shown in Figure 1c, Aβ 1-40 treatment reduced HIPK2 protein levels in HEK-293 cells, compared to vehicle or Aβ 40-1 treatment. Cell treatment with proteasome inhibitor MG132 strongly reduced Aβ 1-40-dependent HIPK2 down regulation, increasing HIPK2 protein levels to an even larger extent when compared to control cells (Figure 1c), suggesting that additional proteasomal degradation processes besides that induced by Aβ 1-40 are likely involved in HIPK2 degradation. Then HEK-293 cells were transfected with HIPK2-Flag and the degradation-resistant HIPK2-K1182R mutant expression vectors. Subsequently cells were treated with Aβ 1-40 or the reverse inactive Aβ 40-1 peptide. As shown in Figure 1d, HIPK2-Flag expression was down regulated by Aβ 1-40 while the reverse peptide was not effective, as reported above for endogenous HIPK2 (Figure 1c); Aβ 1-40 did not affect the expression

of the K1182 mutant (Figure 1d). Altogether, these results show that nanomolar concentrations of soluble Aβ can impair HIPK2 binding to DNA, likely through activation of proteasomal degradation, as assessed by the use of the proteasome inhibitor MG132.

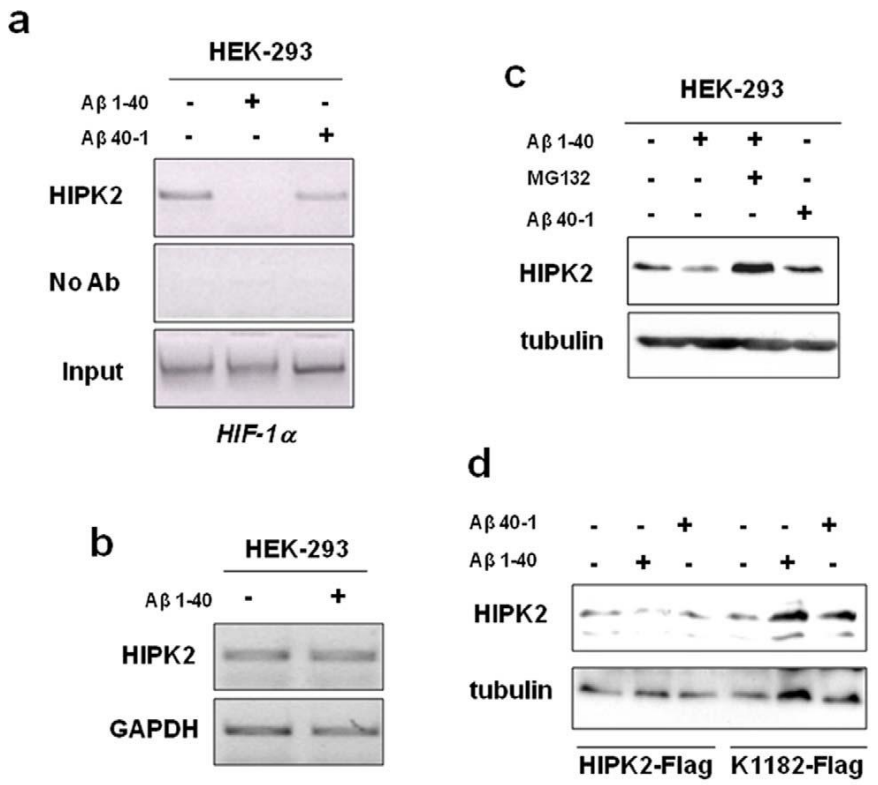

Figure 1. Aβ is responsible for HIPK2 deregulation. (a) ChIP experiments were performed with anti-HIPK2 antibody on HEK-293 cells treated with 10 nM Aβ 1-40 or Aβ 40-1 for 48 h. PCR analyses were performed on the immunoprecipitated DNA samples using specific primers for the human HIF-1α promoter. A sample representing linear amplification of the total input chromatin (Input) was included as control. Additional controls included immunoprecipitation performed with non-specific immunoglobulins (no Ab). A representative experiment of three

independent ones was shown. (b) HIPK2 mRNA expression was determined in HEK-293 cells, treated with 10 nM Aβ 1-40 for 48 h, by reverse-transcriptase (RT)-PCR. GAPDH was used as loading control. (c) Total cell extracts of HEK-293 cells treated with 10 nM Aβ 1-40 for 48 h in the absence or presence of 10 mmol/L MG132 for 6 h were analysed for HIPK2 expression. Anti-tubulin was used as protein loading control. (d) HEK-293 cells were transfected with HIPK2-Flag and the degradation-resistant HIPK2-K1182R mutant expression vector. After transfection, cells were trypsinized, replated in triplicate and treated with 10 nM Aβ 1-40 or the reverse peptide Aβ 40-1 for 48 h. Total cell extracts were analysed by Western immunoblotting with anti-Flag antibody. Anti-tubulin was used as protein loading control.

Endogenous products of APP metabolism negatively affect HIPK2/DNA binding activity

To acquire more insight into the contribution of the different APP processing products to HIPK2 deregulation, we used HEK-293 cells stably transfected with wild type APP751 (HEK-APP) that express high levels of full length APP in comparison with HEK-293 cells [5]. In particular, HEK-APP cells produced and released elevated amounts of Aβ peptides (see Table 1), where Aβ 1-40 was the more abundant isoform (700 pg/mg protein in cellular extracts and 200 pg/mL in the conditioned medium after 48 hrs) than Aβ 1-42 (78 pg/mg in cellular extracts and 42 pg/mL in the conditioned medium after 48 hrs). In order to prevent amyloidogenic APP metabolism, HEK-APP cells were then treated with b secretase inhibitor (bSI); Aβ 1-40 and Aβ 1-42 levels were then measured in both cellular extracts and medium after b secretase inhibition. As shown in Table 1, inhibition of APP amyloidogenic pathway induced by β secretase inhibitor prevented

the formation of Aβ 1-40 and Aβ 1-42 in both the cellular extract and in the medium. HIPK2 binding to HIF-1α promoter was then evaluated in HEK-293 cells and HEK-APP cells that were also treated with the bSI. As shown in Figure 2a (left panel), HIPK2 was easily detected in the HIF-1α promoter in HEK-293 control cells while the recruitment was abolished in HEK-APP cells. Beta-secretase inhibitor treatment enhanced HIPK2 recruitment onto HIF-1α promoter (Figure 2a, left panel), strongly suggesting that APP amyloidogenic metabolites may indeed affect HIPK2 DNAbinding activity. We then tested the ability of the conditioned medium of HEK-APP cells to inhibit HIPK2 DNA-binding. To this aim, HEK-293 cells were cultured with conditioned medium of HEK-APP cells that, as shown in Figure 2a (right panel), abolished HIPK2 binding to HIF-1a promoter. In agreement with our hypothesis, β-secretase inhibitor treatment counteracted the APP-conditioned medium ability to inhibit HIPK2 binding to DNA and re-established HIPK2 recruitment onto HIF-1α promoter (Figure 2a, right panel).

A mechanism through which HIPK2 deregulation may affect p53 conformation could be through metallothionein 2A (MT2A). Metallothioneins can act as potent chelators in removing zinc from p53 in vitro and may modulate p53 transcriptional activity [16]. In particular, HIPK2 depletion has been observed to induce MT2A upregulation, whose inhibition by siRNA restored p53 wildtype conformation and transcriptional activity [17]. These findings suggest that HIPK2 plays a critical role in maintaining p53 wildtype conformation for DNA binding and transcriptional activity indirectly

through MT2A down regulation. Hence, we investigated whether HIPK2 activity to bind MT2A target promoter was somehow compromised in HEK-APP. As shown in Figure 2b (left panel), ChIP assay showed that the HIPK2 recruitment onto MT2A promoter was hampered in HEK-APP cells in comparison with HEK-293 control cells, whereas it was recovered by treatment with β secretase inhibitor. Furthermore, when HEK-293 cells were treated with conditioned medium of HEK-APP cells, we observed an elimination of HIPK2 binding to MT2A promoter (Figure 2b, right panel). Parallel to data on HIF-1α, β secretase inhibitor treatment re-established HIPK2 recruitment onto MT2A promoter thus counteracting the APP-conditioned medium ability to affect HIPK2 binding to DNA (Figure 2b, right panel). These data were supported by the increased HIF-1α-luc and MT2A-luc activities in HEK-APP cells compared to the HEK-293 counterparts (Figure 2c). In agreement, MT2A and HIF-1α mRNA were induced in HEK-APP compared to HEK-293 cells, although to a different extent (Figure 2d). These data suggest that impaired HIPK2 binding to DNA in AD cells correlated with increased HIF-1α and MT2A expression.

	Cell extracts (pg/mg protein)	Medium (pg/ml)
Aβ 1-40 levels	700±6.28	200±9.12
Aβ 1-42 levels	78±5.18	42±5.21
Aβ 1-40 levels after βSI	480±3.17*	128±4.32*
Aβ 1-42 levels after βSI	42±2.12#	28±1.15#

Table 1. Levels of Aβ 1-40 and Aβ 1-42 after treatment with β secretase inhibitor. Levels of Aβ 1-40 and Aβ 1-42 peptides were measured with a commercial ELISA kit in the cellular extracts and conditioned media of HEK-APP cells untreated or treated with b secretase inhibitor at 1 mmol/L for 48 hours. Results are representative of at least three independent experiments 6 S.E.M.

* $p<0.001$ βSI treatment vs corresponding control. # $p<0.01$ βSI treatment vs corresponding control.

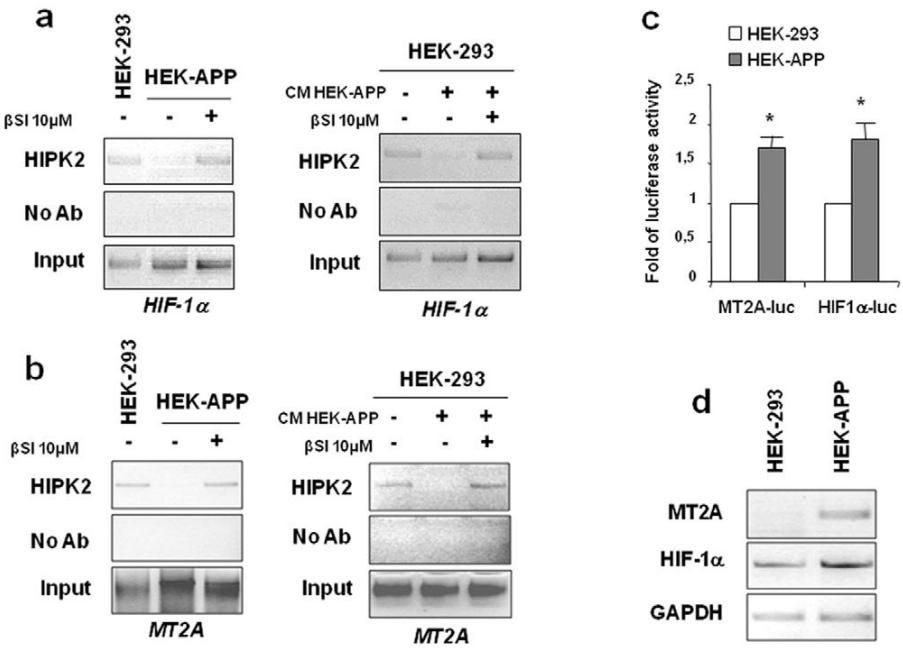

Figure 2. Endogenous products of APP metabolism negatively affect HIPK2 DNA-binding activity. (a) ChIP experiments were performed with anti-HIPK2 antibody on HEK-293 and HEK-APP cells that were also treated with b-secretase inhibitor at 1 mmol/L for 48 h and on HEK-293 treated with conditioned medium from HEK-APP cells for 48 h in the absence or presence of b-secretase inhibitor; PCR analyses were performed on the immunoprecipitated DNA samples using specific primers for the human HIF-1α promoter as shown in Figure 1. (b) ChIP experiments were performer with anti-HIPK2 antibody on HEK-293 and HEK-APP cells that were also treated with b-secretase inhibitor at 1 mmol/L for 4 8h and on HEK-293 treated with conditioned medium from HEK-APP cells for 48 h in the absence or presence of b-secretase inhibitor; PCR analyses were performed on the immunoprecipitated DNA samples using specific primers for the MT2A promoter. (c) HEK-293 and HEK-APP cells were transfected with MT2A-luc and HIF-1α-luc reporter construct and luciferase activity was measured 36 h after transfection. Results normalized to b-galactosidase activity are presented as fold of induction of luciferase activity 6S.D. At least three independent experiments performed in

duplicate. * p,0.01 (Student t-test). (d) MT2A and HIF-1α mRNA expression was determined in HEK-APP compared to HEK-293 cells by reverse-transcriptase (RT)-PCR. GAPDH was used as loading control.

p53 transcriptional activity is restored by zinc

Following the data on MT2A overexpression, we wanted to evaluate whether the p53 dysfunction, related to Aβ exposure in our experimental models, could be restored by zinc supplementation. Thus, p53 transcriptional activity was evaluated by luciferase assay of the p53AIP1-luc apoptotic promoter [18]. HEK-APP cells and the control counterparts were transiently transfected with the p53AIP1-luc reporter plasmid and 24 hrs later treated with 3.4 mM doxorubicin, a cytotoxic agent able to induce DNA damage and apoptosis in a p53-dependent manner [19]. As shown in Figure 3a, p53AIP1-luciferase activity was induced by doxorubicin treatment in HEK-293 cells, whereas it was significantly impaired in HEK-APP cells. Zinc supplementation to HEK-APP cells restored endogenous p53 ability to activate p53AIP1-luciferase reporter in response to doxorubicin (Figure 3a), while it only slightly increased p53 transcriptional activity in HEK-293 cells. Next, analysis of mRNA showed that the doxorubicininduced p53 apoptotic gene transcription (i.e., Bax gene) in HEK-293 cells was impaired after stable transfection of APP751 (HEKAPP) (Figure 3b, compare lane 4 vs lane 2). In agreement with our hypothesis, zinc supplementation to HEK-APP cells restored druginduced Bax transcription (Figure 3b, compare lane 4 vs lane 5). Finally, Western immunoblotting showed

doxorubicin-induced Bax expression in HEK-APP cells only after zinc supplementation (Figure 3c). These data suggest that the Aβ-inhibited wild-type 53 apoptotic transcriptional activity, in response to drug, was reactivated by zinc.

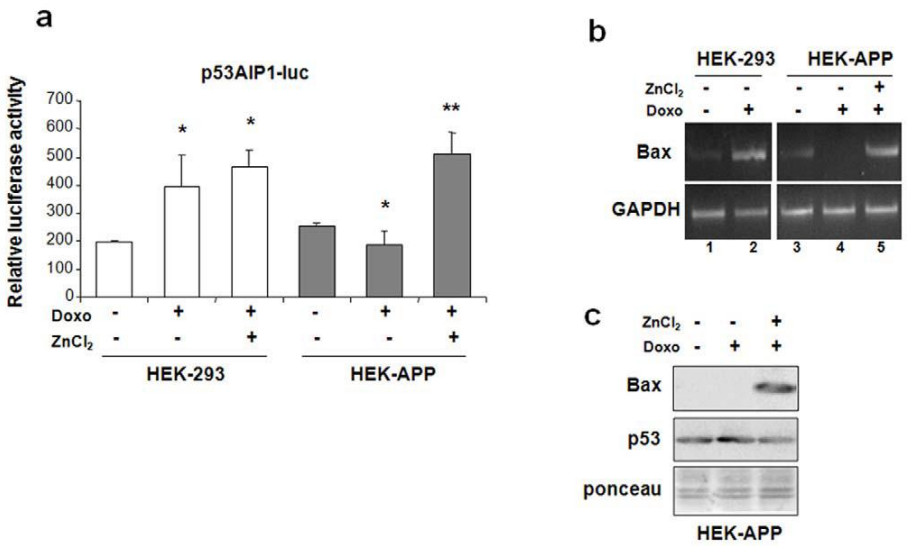

Figure 3. Zinc supplementation to HEK-APP restores p53 pro-apoptotic transcriptional activity. (a) HEK-293 and HEK-APP cells were transfected with p53AIP1-luc reporter construct and 24 h after transfection treated with doxorubicin (3.4 mM) and zinc (100 mM) for 24 h before luciferase activity was assayed. Results normalized to b-galactosidase activity are shown as relative luciferase activity 6S.D. At least three independent experiments performed in duplicate. * $p<0.05$ vs HEK-293 or HEK-APP; ** $p<0.01$ vs HEK-APP (Bonferroni Multiple Comparison test). (b) Bax mRNA expression was determined in HEK-APP compared to HEK-293 cells by reverse-transcriptase (RT)-PCR after treatment with doxorubicin (3.4 mM) and zinc (100 mM) for 24 h. GAPDH was used as loading control. (c) Total cell extracts of HEK-APP cells treated with doxorubicin (3.4 mM)

and zinc (100 mM) for 24 h were analysed for Bax and p53 expression. Protein loading control was shown as Ponceau staining.

AD fibroblasts show conformationally altered p53 protein and a reduced HIPK2 DNA-binding activity that are restored by zinc

The conformational status of p53 was analyzed in fibroblasts derived from AD and non-AD subjects by immunoprecipitation technique using two conformational-specific antibodies, PAb1620 and PAb240, which discriminate folded versus unfolded p53
tertiary structure [16]. Fibroblasts from AD patients from our cell repository express high levels of unfolded p53, as shown by Uberti et al. [7] and replicated in the two representative cell lines used in the current experimental setting (Figure 4a). Zinc supplementation to AD fibroblasts strongly reduced p53 mutant-like conformation (Figure 4a, upper panel), as is also shown by quantitative analysis of p53 immunoprecipitates (Figure 4a, lower panel). Analysis of mRNA showed that MT2A expression was upregulated in AD cells (Figure 4b), suggesting that HIPK2 deregulation might be involved in p53 misfolding in AD cells, likely through MT2A upregulation, as previously shown [17]. As on HEK-APP cells, we then investigated whether HIPK2 activity to bind target promoters was somehow compromised in AD fibroblasts. As shown in Figure 4c, ChIP assay showed that the HIPK2 recruitment onto HIF-1α promoter was present in fibroblasts from control subjects, whereas it was hampered in AD cells. Of note, zinc treatment to AD fibroblasts restored HIPK2 binding activity to DNA, likely counteracting the proteasomal

degradation, as previously reported [20] and thus suggesting that zinc was able to affect the dual role of HIPK2 as DNA co-repressor and as p53 activator.

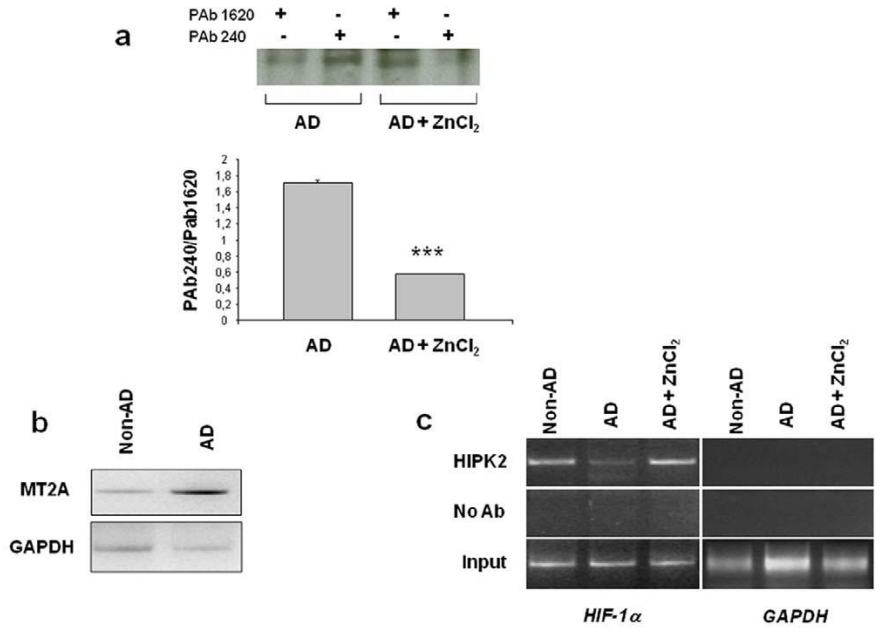

Figure 4. Fibroblasts from Alzheimer patients show misfolded p53 with increased MT2A expression and reduced HIPK2 binding to DNA. (a, upper panel) Equal amounts of total cell extracts from fibroblasts derived from AD patients were treated with 100 mM zinc for 16 h and then immunoprecipitated with anti-p53 conformational antibodies, PAb1620 for wild-type conformation and Pab240 for mutant-like conformation.

Immunoprecipitates were analysed by Western immunoblotting with the polyclonal anti-p53 antibody. A representative experiment of three independent ones was shown. (a, lower panel) Densitometric analysis of immunoprecipitated p53 as above, showing reduction of PAb240 mutantlike conformation after zinc treatment. *** $p<0.0001$ vs AD (Student t-test). (b) MT2A expression was determined in

fibroblasts derived from AD patients compared to non-AD patients by reverse-transcriptase (RT)-PCR. GAPDH was used as loading control. (c) ChIP experiments were performed with anti-HIPK2 antibody on fibroblasts from AD and non-AD patients. PCR analyses were performed on the immunoprecipitated DNA samples using specific primers for the human HIF-1α promoter. A sample representing linear amplification of the total input chromatin (Input) was included as control. Additional controls included immunoprecipitation performed with non-specific immunoglobulins (no Ab). A representative experiment of three independent experiments was shown. Amplification of GAPDH promoter was used as control of HIPK2 binding specificity to the HIF-1α promoter.

DISCUSSION

For the first time, we can describe a link between Aβ, AD related conformationally altered p53 and HIPK2, a transcriptional co-repressor and activator of p53 apoptotic function. We previously demonstrated the existence of an unfolded state of p53 protein in fibroblasts from AD patients that led to an impaired and dysfunctional response to stressor [6,21]. Here we examined the molecular mechanisms underlying the impairment of p53 activity in two cellular models, HEK-293 cells overexpressing the amyloid precursor protein and fibroblasts from AD patients, starting from recent findings showing that p53 conformation may be regulated by HIPK2 [13]. Our data suggest that Ab peptides may be responsible for HIPK2 deregulation. This is supported by the observation that Ab peptides down-regulated HIPK2 expression via proteasomal degradation (Figure 1c and 1d) leading to HIPK2 disappearance from target promoters such as HIF-1α and MT2A (Figure 1a, 2a and 2b). In

agreement, HIF-1α and MT2A mRNA upregulation was found in HEK-APP cells that overexpress APP751 (Figure 2d). The induction of MT2A, depending on HIPK2 knockdown has been reported to be responsible for p53 misfolding and inhibition of p53 transcriptional activity [16]; therefore, the present data suggest that HIPK2 deregulation in HEK-APP cells and fibroblasts from AD patients might be involved in p53 misfolding, most likely through MT2A upregulation.

Attempting to better investigate the contribution of APP metabolic products in the modulation of HIPK2 expression and change in p53 conformational state, we then used HEK cells with wild-type APP able to generate high levels of Aβ 1-40 and Aβ 1-42 both intracellularly and secreted in the medium, with Aβ 1-40 about 10 times more abundant than Aβ 1-42 [5]. We found that reducing APP amyloidogenic metabolism by treating HEK-APP cells with a β-secretase inhibitor prevented the deregulation of HIPK2 (Figure 2a, 2b) and likely the generation of the unfolded p53 isoform. Interestingly, we previously demonstrated that asecretase inhibitor did not affect unfolded p53 isoform and did not modify the cellular response to doxorubicin [5]. It is worthy to note that the conditioned medium of HEK-APP cells was able to affect HEK-293 cells recapitulating the HEK-APP phenotype, at least in terms of HIPK2 DNA-binding and altered p53 conformational changes. The Aβ peptides released in the media by HEK-APP appeared to trigger such effects. In fact, the conditioned media of HEK-APP cells pretreated

with β secretase inhibitor, were unable to inhibit HIPK2 binding to HIF-1α and MT2A promoters, in HEK-293 cells (Figure 2b).

HIPK2 has been shown to be down-regulated during hypoxia by Siah2-induced proteasomal degradation [22]. Moreover, HIPK2 impairment during hypoxia induces de-repression of target genes such as HIF-1α, and inhibition of p53 activity [20]. Zinc supplementation to hypoxia-treated cells restores HIPK2 stability and binding to HIF-1α promoter, rescuing also the p53 apoptotic transcriptional activity [20]. Therefore, discovering the mechanisms of HIPK2 inhibition and the ways to manipulate HIPK2 activity is an interesting option to affect several biological pathways [23]. Here we showed a novel mechanism of HIPK2 down-regulation mediated by Aβ, likely through activation of proteasomal degradation. HIPK2 is an unstable protein that is degraded via the proteasome pathway induced by several E3 protein ligases [24], although the molecular mechanisms underlying HIPK2 proteasomal degradation in conditions related to Ab exposure deserve further studies.

The deregulation of HIPK2 function was further confirmed in fibroblasts from sporadic AD subjects, an extra-neuronal model showing a number of abnormalities in metabolic and biochemical processes, with some of them mirroring events that occur in the AD brain [25]. We found that fibroblasts from AD patients are characterized by a decreased HIPK2 DNA-binding activity, besides showing a conformationally altered p53. Since one of the features that distinguishes AD from non-AD fibroblasts is a defective non-amyloidogenic APP processing, likely favouring an aberrant Aβ

peptides production [26], these data suggest that this abnormality may be, at least in part, responsible for altered HIPK2 binding to promoters. HIPK2 is ubiquitously expressed and has been found in developing neurons [27]. HIPK2 overexpression suppresses Brn3a-dependent transcription of brn3a, trkA and bcl-xL resulting in apoptotic cell death in cultured sensory neurons [27,28]. Moreover, HIPK2 is an important component in the TGFb signalling pathway that regulates the survival of midbrain dopamine neurons, as suggested by HIPK2 knock-out mice [28,29]. Interestingly, it has been recently shown that HIPK2 is required for the trakA to p75NTR transition that leads to increased generation of Aβ that accompanies aging [30], suggesting a regulatory loop that tends to inhibit HIPK2 during aging contributing to AD.

The impairment of HIPK2 function, by shifting p53 protein structure from a wild-type to a conformationally altered phenotype, should increase the threshold to a noxious stimulus, as reported [13]. Therefore, we tested, in HEK-APP cells, whether the reduced efficacy of doxorubicin-induced apoptosis was due to an impairment of p53 apoptotic activity. In agreement with our hypothesis, we found that these cells showed a reduced HIPK2 DNA binding activity and Bax transcription in response to doxorubicin that was rescued by zinc (Figure 3). Zinc has been reported to restore p53 function in HIPK2 depleted cells [13,17]. The capability of zinc to act in cells is ascribed to the existence of zinc transporters, that are required to convey this ion across cellular membranes, since zinc is unable to passively diffuse across cell membranes [31]. The use of zinc for AD treatment

is controversial, since several recent works reported the capability of zinc to cause the precipitation of Aβ into nonfibrillar amorphous aggregates [32]. However, in our experiments we speculate that the capability of zinc supplementation to restore the Aβ 1-40-inhibited HIPK2 DNA-binding appears not to be associated to a metal ion-induced precipitation of the synthetic peptide but rather to counteracting a degradation mechanism. This is supported by the observations in fibroblasts from AD patients, in which zinc treatment was able to rescue HIPK2 binding to its target promoters. However, we could also not exclude the hypothesis that zinc may be able to withdraw the synthetic peptide from cellular environment thus modulating its interaction.

In summary, we hypothesize that low amounts of soluble Aβ, not resulting in cellular toxicity, may be responsible for important modulatory effects at cellular level before triggering the amyloidogenic cascade. For the first time we found that one of these modulatory effects may be the inhibition of HIPK2 activity, with MT2A upregulation, in turn responsible for the induction of an altered conformational state of p53. As a result of this conformational change, p53 lost its transcriptional activity and was unable to properly activate an apoptotic program when cells were exposed to a noxious stimulus. Altogether, Aβ-induced HIPK2 depletion and unfolded p53 may contribute to AD pathogenesis leading to dysfunctional cells. The definition of this new target is useful to help characterize the hierarchical scale of events driven by beta-amyloid so as to better understand the pathogenesis of AD. Furthermore, the recognition of

HIPK2 as new target of the effect of Aβ could suggest a new putative functional biomarker useful in addressing new therapeutic strategies.

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Part 2.

Highlight on the potential relevance of HIPK2 as new target for Alzheimer's disease

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UNFOLDED p53 IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE: IS HIPK2 THE LINK?

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ABSTRACT

p53 transcriptional activity depends mainly on posttranslational modifications and protein/protein interaction. Another important mechanism that controls p53 function is its conformational stability since p53 is an intrinsically unstable protein. An altered conformational state of p53, independent from point mutations, has been reported in tissues from patients with Alzheimer's disease (AD), leading to an impaired and dysfunctional response to stressors. Recent evidence shows that one of the activators that induces p53 posttranslational modification and wild-type conformational stability is homeodomain-interacting protein kinase 2 (HIPK2). Hence, conditions that induce HIPK2 deregulation would result in a dysfunctional response to stressors by affecting p53 activity. Discovering the mechanisms of HIPK2 activation/inhibition and the ways to manipulate HIPK2 activity are an interesting option to affect several biological pathways, including those underlying AD. Soluble beta-amyloid peptides have recently been involved in HIPK2 degradation, in turn regulating the p53 conformational state and vulnerability to a noxious stimulus, before triggering the amyloidogenic cascade. Here we discuss about these findings and the potential relevance of HIPK2 as a target for AD and highlight the existence of a novel amyloidbased mechanism in AD potentially leading to the survival of injured dysfunctional cells.

INTRODUCTION

Alzheimer's disease (AD) is a primary progressive neurodegenerative disease where the aberrant metabolism of the amyloid precursor protein (APP) and the production and deposition of beta-amyloid peptide (Aβ) are considered responsible for neuronal death [1]. A putative link between the tumor suppressor p53 and the perturbation of APP metabolism has been demonstrated. In particular, an altered protein conformational state of p53, independent from point mutations, has been reported in tissues from patients with AD that led to an impaired and dysfunctional response to stressors [2-4]. One of the activators that induces p53 posttranslational modification and wild-type conformational stability is homeodomain-interacting protein kinase 2 (HIPK2) [5]. Here we discuss about the potential relevance of the definition of HIPK2 as a target for AD and highlight the existence of a novel amyloid-based pathogenetic mechanism in AD involving HIPK2 and unfolded p53, potentially leading to the survival of injured dysfunctional cells.

Alzheimer's disease and beta-amyloid

Beta-amyloid in AD is the result of the proteolytic metabolism of APP, an integral cell membrane glycoprotein of 697-770 residues which is the substrate of three proteolytic enzymes in two alternative pathways mutually in equilibrium [6]. In the nonamyloidogenic pathway, a protease, named α-secretase, cleaves APP in the extracellular domain and releases the ectodomain of APP (soluble APPα) into the extracellular space, thus precluding Aβ formation.

Otherwise, in the amyloidogenic pathway, Aβ is formed following cleavage by β and γ secretases, that cleave the N and C terminus of Aβ, respectively (Figure 1). The two main isoforms found in AD brains are Aβ 1-40 and Aβ 1-42. Physiologically the 40-amino-acid long peptide is the most abundant form [7-9], since the concentration of secreted Aβ 1-42 is about 10% that of Aβ 1-40 [10]. For these reasons, Aβ1-40 and Aβ1-42 may have different biological actions [11] and the ratio of their production may be altered in pathological conditions, such as in familial AD [12].

Although the direct and the indirect neurotoxic role of Aβ are unchallenged (for an exhaustive review see [13]), recent findings suggest that the peptide may have so far unforeseen physiological roles [14]. Besides its presence in AD brains, experimental evidence indicated that Aβ peptides are produced constitutively by all cells, including neurons, and are found in the nM-pM range in the CSF of non-demented individuals [15] and in media from neuronal and non-neuronal cell cultures [16, 17], thus suggesting that, as well as having a potential pathological role in AD, Aβ peptides under normal conditions may have a role in the regulation of physiological functions, consistent with their ubiquitous presence and normal synthesis. We will discuss here on how Aβ may have a role in the regulation of the function of p53.

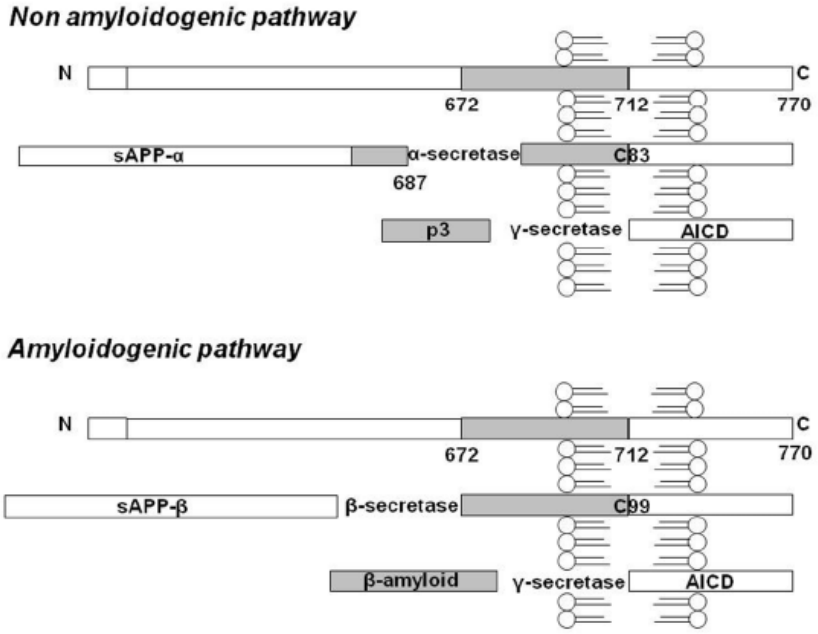

Figure 1. APP metabolism: schematic representation of the non-amyloidogenic and amyloidogenic pathway. Here the 770 residue APP processing is schematized, even if the 695 and 751 transmembrane forms of APP exist. In the non-amyloidogenic pathway, α-secretase cleaves APP in the extracellular domain and releases soluble APPα into the extracellular space. Following this cleavage, a second enzymatic product, the C-terminal fragment (αCTF or C83), which can be a substrate for γ-secretase, yields a non-amyloidogenic 3 kDa fragment known as p3. In the amyloidogenic pathway Aβ is formed following cleavage by β and γ secretases, respectively. The cleavage of APP at the residue 1 of Aβ sequence results in a truncated form of sAPP (sAPPβ) and in a C-terminal fragment of 12 kDa (βCTF or C99). The final step in the amyloidogenic pathway is the cleavage of βCTF, to liberate Aβ by γ-secretase. Furthermore, in both the amyloidogenic and non-amyloidogenic pathways, the cleavage of C83 and C99 fragments by γ-secretase also results in the generation of C-terminal peptides of 57-58 residues, referred as APP intracellular domain (AICD).

p53 function and its role in aging and neurodegeneration

From its discovery in 1979 [18], p53 continues to fascinate scientists and it is still nowadays one of the most extensively studied protein. Such interest is due to the key role of p53 in the prevention of cancer so as to be defined the "guardian of the genome" [19]. However, activities of p53 might have a role not only in regulating cancer progression, but also in the control of other aspects of health and disease such as development, aging and metabolism [20]. p53 exerts its main biological role of tumor suppressor and master controller of the genomic integrity especially acting as transcription factor [21]. p53 oversees the correct implementation of processes and it intervenes only in case of dangerous deviations from the proper cellular activity. When the cell is exposed to critical conditions or undergoes damages p53 arbitrates cell faith [22]. Loss of p53 or deregulation of its activities leads not only to cancer but also to cardiovascular, metabolic diseases, neurodegeneration and to the process of aging, because of the great number of p53-regulated genes which underlie all these different biological events [23] (see Table 1).

The very first signal which stimulates p53 activity is the DNA damage and various genotoxic insults that could constitute a danger to the genomic integrity of cells such as oxidative stress, DNA damage, hypoxia, oncogene activation, telomere erosion, changes in metabolism, unusual prolongation of some signaling pathways and local depletion of nutrients, among others [41]. Immediately after these damages p53 is the substrate of a great number of possible posttranslational modifications introduced by a variety of specific

enzyme systems [42]. These alterations comprise a large network of covalent changes inducing characteristic modifications within the protein quantity, activity and ability to interact or cooperate with a variety of other proteins [43]. The delicate balance between p53 Structure and Activity Relationship (SAR) can be disrupted even by a single amino acid substitution within the DNA binding domain (DBD) which is sufficient to limit or abolish the capacity of p53 to direct sequence-specific transcriptional activity [44]. This is the case of the majority of human cancers in which missense mutations in the DBD result in an altered network which can affect the prognosis. Beside gene mutation, p53 activity could be impaired also as consequence to a conformational change. p53 may lose its transcriptional activity due to an unfolded tertiary structure which determines a reduction in its affinity for specific DNA target sequence. Recent observations confirm that p53 structure changes can play a central role in aging and in AD [45, 46].

Because of its role in establishing senescence and in determining organism aging when its activity is increased, p53 can promote selected aspects of the aging process. Different studies indicate a delicate balance between the tumor suppressive and age promoting functions of p53. In several mouse models and also from human population studies, alteration of p53 activity has been demonstrated to influence the comparison of premature/accelerate aging under some circumstances (such as stress) or otherwise induce tumor suppression [47-52]. Evidence in mice and humans suggest that p53 acts as a longevity-assurance gene, basically reducing the influence of

tumorigenesis [53, 54]. Our group has studied p53 in fibroblasts from aged controls and demented patients finding that with aging there is an increase in the expression of an unfolded protein state, which is more pronounced in AD patients and is not dependent on gene mutations [55].

As a result of such conformational change, p53 partially loses its activity and shows a significant impairment in its DNA binding and transcriptional capacity when cells are exposed to a noxious stimulus [55]. In fact, AD fibroblasts are less vulnerable to oxidative injury than fibroblasts from non-AD subjects to the point that conformationally altered p53 has been proposed as putative biomarker for early AD [55]. This altered conformation can be due to a loss of zinc (Zn^{2+}) ion in the core domain of the protein, that provides the basic scaffold for the DNA binding and which has been demonstrated to be crucial for the stabilization of p53 in the so called "wild-type" folded form. Exposure of wild-type p53 (wt53) to metal chelators such as ethylenediaminetetraacetic acid (EDTA) or orthophenanthroline determines a rapid switch to the unfolded form positive to an antibody (clone PAb240), recognizing a primary epitope cryptic in wt53 [56].

Upon addiction of micromolar amounts of Zn^{2+}, the protein undergoes a refolding to the native form and reacquires DNA-binding competence [56]. Trying to investigate the cause of this alteration we found that the exposure to nanomolar concentrations of the betaamyloid peptide 1-40 (Aβ 1-40) induced the expression of the unfolded p53 protein isoform in fibroblasts derived from non-AD

subjects [3]. These data suggested that the tertiary structure of p53 and the sensitivity to p53-dependent apoptosis is influenced by low concentrations of soluble Aβ. On this basis, we assumed that low amounts of soluble Aβ induce early pathological changes at cellular level that may precede the amyloidogenic cascade and one of these changes is the induction of the unfolded state of p53, suggesting a role of the protein in the early pathogenesis of AD [3]. Recently, in cultured peripheral blood cells derived from AD patients we and others observed a detectable amount of unfolded p53, recognized with the antibody PAb240, which made these cells distinct from those of controls. We suggest that unfolded p53 could be used as a biomarker of the disease also in early stages [57, 58]. Zhou and collaborators speculate that unfolded p53 might be the responsible for the failure of G1/S transition checkpoint in AD lymphocytes, which is normally mediated by wt53, connecting unfolded p53 to a peripheral event associated to the disease. They suggest that the cause of p53 conformational change could be oxidative stress, Aβ toxicity and the effects of oxygen free radicals [59]. This additional observation about the existence of an altered state of p53 at the peripheral level in subjects with AD reinforces the hypothesis that the protein can have a role in the pathogenesis of the neurodegenerative disease. However, further studies are needed to understand the causes of such conformational change and, as consequence, about how unfolded p53 contributes to the progress of age and neurodegeneration.

Cellular outcome	p53 gene target	Cellular stress	Molecule modifier	p53 residue and type of modification	Reference
Cell cycle checkpoint	p21	Mild DNA damage	PCAF	Lys320-Acetylation	24
	Gadd45	Mild DNA damage	E4F1	Lys320-Ubiquitylation	25
		UV radiation	CK2	Ser392-Phosphorylation	26, 27
Apoptosis	Transactivation: Bax, Bcl-X1, Apaf-1, Fas, Bad, Noxa, Puma Transrepression: Bcl-2, Bcl-X1, Survivin	Severe DNA damage	HIPK2	Ser46-Phosphorylation	28, 29, 30, 31
		Severe DNA damage	CBP	Lys382-Acetylation	28
		UV radiation	MAPK	Ser46-Phosphorylation	32
		Genotoxic stress	DYRK2	Ser46-Phosphorylation	33
		Genotoxic stress	PKCδ	Ser46-Phosphorylation	34
		Severe DNA damage	MOF and TIP60	Lys120-Acetylation	35, 36
		Severe DNA damage	p300	Ls373-Acetylation	37
Senescence	p21 p66	DNA damage	PML IV	Lys382-Phosphorylation	38
		DNA damage	PML IV	Ser20-Phosphorylation	38
		Senescence stresses	ATM/Chk2	Ser15, Ser20-Phosphorylation	32, 39
		Senescence stresses	ATR/Chk1	Ser15, Ser37-Phosphorylation	32, 39, 40

Table 1. p53 at the crossroad of complex networks of stress response pathways. Different intercellular and extracellular stresses result in cellular outcomes directly mediated by p53 activation. The activation of p53 passes through a variety of modifications that occur at the protein level; these post-translational modifications are crucial in regulating p53 function. We summarize in the table p53 signalling transduction pathways resulting in activation of specific downstream gene targets, whose role is to drive cell destiny.

Misfolded p53 and the role of HIPK2

Homeodomain-interacting protein kinase 2 (HIPK2) is a member of a novel family of nuclear serine/threonine kinases that localizes into the nuclear bodies and acts as co-repressor for several transcription factors [60]. Furthermore, one important function of HIPK2 is the apoptotic activation of p53 in response to genotoxic agents [5]. HIPK2 interacts physically and functionally with p53 phosphorylating it at Serine 46 (Ser46) for apoptotic activation (Table 1) [28-31]. HIPK2 interacts also with the acetyltransferase CREB binding protein (CBP) and co-localizes with CBP and p53 at promyelocytic leukemia nuclear bodies (PML-NBs); here HIPK2-mediated p53Ser46 phosphorylation enhances CBP-mediated p53 acetylation at Lys382, potentiating the expression of pro-apoptotic target genes [28]. Thus, although Ser46 can be phosphorylated by additional kinase other than HIPK2, including ATM [61], DNA-dependent protein kinase (DNA-PK) [62], protein kinase C δ (PKCδ) [63], and dual-specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) [64], the fact that only HIPK2 can drive Lys382 acetylation renders this kinase a unique and complex regulator of p53 apoptotic function. Thus, in the absence of HIPK2, the lack of Lys382 acetylation strongly impairs p53 pro-apoptotic activation [65]. HIPK2 function is important for the p53 acetylation/deacetylation balance by regulating the activity of deacetylase Sirt1, through repression of NADPH oxidase 1 (Nox1) [66]. Thus, in the absence of HIPK2, oxygen reactive species (ROS) are induced in cancer cells, with activation of Nox1 and Sirt1 activities that inhibit p53 apoptotic activity in response to DNA

damage [66]. The role of HIPK2 in p53 activation in cancer cells involves also wt53 protein conformation. In the absence of HIPK2, p53 acquires a misfolded conformation loosing DNA binding and transcriptional activities, depending on deregulation of metallothioneins and Zn2+ [67, 68]. Thus, Zn2+ supplementation to HIPK2 depleted cancer cells determines a regain of the wt53 protein conformation and restoration of DNA binding and transcriptional activities in response to genotoxic agents *in vitro* and *in vivo* [67]. Treatment of mice carrying tumors derived from HIPK2-depleted cells with a combination of Zn2+ and chemotherapeutic drug Adriamycin enhances growth suppression of such tumors *in vivo* [67]. From these data it appears that HIPK2 plays a major role in the regulation of p53 function through the switch between p53 dynamic conformational states, and that Zn2+ is a fundamental cofactor. The binding and exchange/transport of Zn2+, as well as of other heavy metals, such as cadmium or copper, are modulated by metallothionein (MT), a family of at least 10 highly conserved, low molecular weight cystein-rich metalloproteins [69]. The interest in MTs derives from their role as regulators of p53 folding and activity, since small amount of MTs can induce p53 activity regulating the folding of the DBD domain through Zn2+ modulation, whereas excess of MTs reduces p53 activity by exerting their Zn2+ chelator function [70, 71]. Furthermore, an increase of MTs expression also correlates with chemoresistance, increased cell proliferation, reduced apoptosis and inhibition of p53 activity in various human tumors [72]. In this regard, it has been shown that HIPK2 negatively regulates MT2A

gene, whose mRNA transcript isoform appears to be associated with cell proliferation in invasive ductal cancer tissues and that, on the contrary, HIPK2 depletion correlates with MT2A up-regulation in MCF7 breast cancer cells [68]. Moreover, MT2A depletion by siRNA (silencing RNA) in cells depleted of HIPK2, restores wt53 conformation [68].

HIPK2 inactive/active switch in Alzheimer's disease and the relevance of zinc supplementation

Given the role of HIPK2 in maintaining wt53 conformation in tumor cells and the presence of an unfolded state of p53 in AD peripheral cells, the interest of a putative modulation of HIPK2 in AD type dementia has been investigated. As reported above, the exposure to nanomolar concentrations of Aβ led to an increased content of unfolded p53 protein in fibroblasts from AD patients, compared to control subjects [3]. Looking at the molecular mechanism of p53 misfolding, a link among Aβ, p53 and HIPK2 in the neuropathology may be proposed [73]. Aβ has been hypothesized to be responsible for HIPK2 proteasomal degradation, in turn resulting in HIPK2 nuclear disappearance from target promoters such as hypoxia inducible factor 1α (HIF-1α) [74] and MT2A [68], whose mRNA was found upregulated in cellular models of AD [73]. The induction of MT2A, depending on HIPK2 knockdown, has been reported to be responsible for p53 misfolding and inhibition of p53 transcriptional activity [68]. On this basis, we could speculate that, in AD, HIPK2 deregulation might be involved in p53 misfolding, most likely

through MT2A upregulation. Data from literature point out that MTs play a very important role in controlling Zn2+ homeostasis. Increased MTs levels induce down-regulation of many biological functions related to Zn2+, such as metabolism, gene expression and signal transduction [75]. The MTs can serve as a source of Zn2+ and are considered also as strong antioxidants and protective factors against stress conditions [76]. MTs are highly expressed in both astrocytes and hippocampal neurons in the aging brain and are a key area of investigation for inflammation and modulation of Zn2+ availability in the aging brain [77]. Therefore these proteins are being intensively studied in the context of aging and longevity mechanisms [78]. During aging there is an increased expression of MTs mRNA but decreased levels are found in healthy centenarians, indicating a possible selection for survival of low expressors [79]. However the precise function of these proteins in aging is still debated, because their protective role could also be deleterious in the case of sequestration of Zn2+ as observed both in human aging and AD [73, 80, 81]. The high MTs mRNA in lymphocytes from old people and Down syndrome subjects and the low MTs mRNA in lymphocytes from young adult and centenarians are a significant support to the idea of a pleiotropic role of MTs in aging [82]. Therefore, the role of HIPK2 in MT2A regulation could unveil interesting interplay between these molecules with p53 also in other chronic diseases such as AD. Taking into account MT2A upregulation dependent on HIPK2 depletion, zinc supplementation to hypoxiatreated cancer cells reestablishes HIPK2 nuclear localization and DNA binding activity,

restoring p53 apoptotic function in response to anticancer drug [83]. Similarly, zinc-supplementation to AD cellular models restores HIPK2 DNA binding, p53 wild-type conformation and apoptotic activity in response to a genotoxic agent [73]. Hence, we could define that, in AD, HIPK2 plays a critical role in maintaining p53 wild-type conformation indirectly through MT2A down regulation, and that Zn2+ is a fundamental cofactor. On the basis of data here summarized, we speculate that soluble Aβ may be responsible for important modulatory effects at cellular level before triggering the amyloidogenic cascade. One of these modulatory effects may be the inhibition of HIPK2 activity, with MT2A upregulation, in turn responsible for the induction of an altered conformational state of p53. As a result of this conformational change, p53 loses its transcriptional activity and is unable to properly activate an apoptotic program when cells are exposed to a noxious stimulus. Altogether, Aβ-induces HIPK2 depletion and the consequent unfolded p53 may contribute to AD pathogenesis leading to dysfunctional cells (Figure 2).

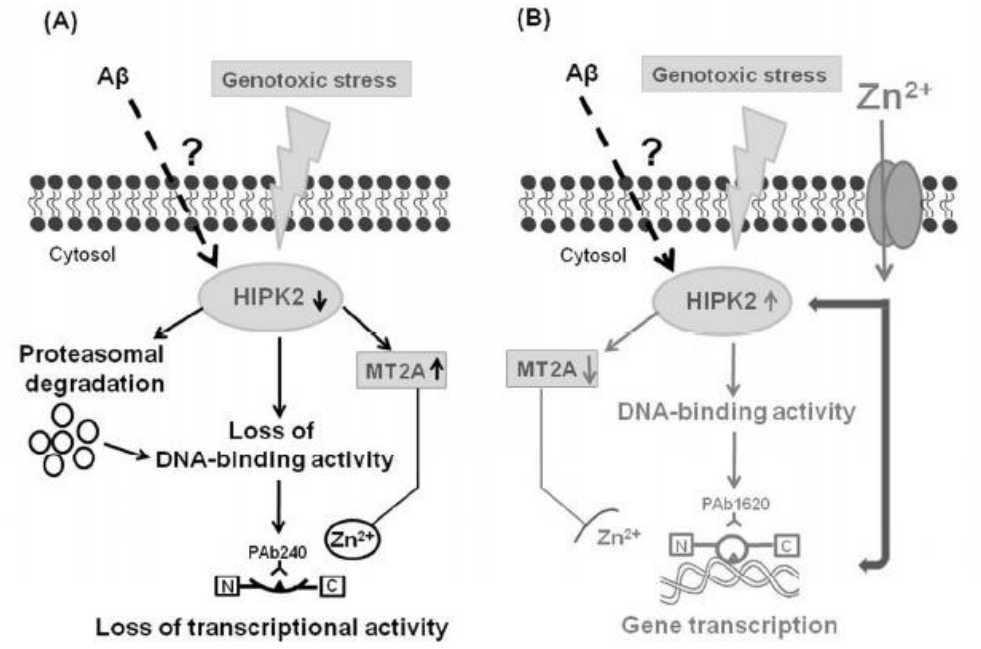

Figure 2. Working hypothesis for a putative link among p53 protein, soluble Aβ and HIPK2. The figure shows a novel mechanism of HIPK2 deregulation mediated by Aβ. HIPK2, when activated in response to DNA damaging agents, is able to interact physically and functionally with p53 and phosphorylate p53 at serine 46, thus regulating p53-induced apoptosis. HIPK2 also acts as transcriptional corepressor and deregulates the promoter metallothionein 2A (MT2A). MT2A may regulate p53 activity inducing protein folding through zinc modulation. In the presence of soluble Aβ, HIPK2 expression and activity are inhibited through Aβ-induced degradation via the proteasome system (panel **A**). HIPK2 deregulation results in the induction of MT2A (panel **A**), that exerts its Zn2+ chelator function. As a consequence, p53 protein misfolding (changing the wild-type conformation to a conformationally altered status) with subsequent abolishment of wild type p53 DNA binding and transcriptional activity occurs (panel **A**). Zinc supplementation counteracts Aβ effects on HIPK2 regulation (panel **B**). Zinc enters into cells through specific zinc transporters, that are required to convey this ion across cellular

membranes, since zinc is unable to passively diffuse across cell membranes. Zinc can directly restore p53 function (panel **B**). In addition, zinc can also affect HIPK2 function, thus resulting in HIPK2 reactivation (panel **B**). As consequence, MT2A is deregulated and p53 conformational can switch to the wild-type and transcriptional active form (panel **B**).

CONCLUSIONS

In humans, aging may be influenced by the balance of cell survival versus cell death, a decision at least in part regulated by checkpoints proteins, by preservation of DNA integrity and correct repair [84]. We focused mainly on one of such proteins, p53, recently shown to be involved in aging and AD [55, 57, 58]. A link between AD pathology and an unfolded state of p53 has been proposed, based on findings that with aging an increase of unfolded p53 occurs in healthy subjects and is peculiarly high in AD patients. By investigating what could be the contribution of a conformational change of p53 to AD pathogenesis, for the first time we define a hierarchical scale of events driven by Aβ: Aβ-induced HIPK2 depletion and unfolded p53 may contribute to AD pathogenesis leading to dysfunctional cells [73]. This observation is intriguing in light of recent data showing that p53 suppresses cellular aging. In this context, despite the well-known capability of p53 to induce senescence, more recent evidence demonstrated that p53 can also act as a suppressor of cellular senescence while promoting cell cycle arrest [85]. This dual effect on senescence may be ascribed to the fact that p53 regulates cell growth and metabolic stress through different pathways [86]. One of these is

represented by mTOR pathway, which is strictly connected with organismal aging, as its inhibition may be one of the main mechanisms decreasing lifespan [87]. p53 is able to regulate activity of mTOR following DNA damage or oncogenic stress by activation of specific effectors (PTEN, AMP kinase and TSC-2), each of which signals to diminish the activity of mTOR, which is involved in senescent phenotype [85, 88]. By suppressing mTOR, p53 can suppress senescent phenotype, converting senescence into quiescence [89]. Furthermore, mTOR inhibition induces autophagy, thus resulting in the accumulation of protein aggregates, endoplasmic reticulum stress and mitochondrial dysfunction, each of which could promote senescence [86]. Thus, taking into account that with aging an increase of unfolded p53 occurs, the loss of wild-type p53 conformation could free mTOR, thus inducing aging-associated abnormalities.

Thirty years have passed since p53 discovery and in these decades a lot of information about its structure, functions and pathways has been achieved. In the fourth decade of p53 investigation the research community hopes to be able to get new drugs to affect p53 function to treat not only cancer but also important neurological conditions, such as AD. The recognition of HIPK2 as new target of the effect of Aβ could suggest a new putative functional biomarker useful in addressing new therapeutic strategies [90].

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Papers published as result of Doctoral work

(from 2007 to 2010)

1. **Stanga S**, Lanni C, Govoni S, Uberti D, D'Orazi G, Racchi M. *"Unfolded p53 in the pathogenesis of Alzheimer's disease: is HIPK2 the link?"*. Aging (Albany NY) 2010; 2(9): 545-54.

2. Lanni C, Nardinocchi L, Puca R, **Stanga S**, Uberti D, Memo M, Govoni S, D'Orazi G, Racchi M. *"Homeodomain interacting protein kinase 2: a target for Alzheimer's beta amyloid leading to misfolded p53 and inappropriate cell survival"*. PLoS One. 2010; 14;5(4):e10171.

3. Lanni C, Racchi M, **Stanga S**, Mazzini G, Ranzenigo A, Polotti R, Memo M, Govoni S, Uberti D. *"Unfolded p53 in blood as a predictive signature signature of the transition from mild cognitive impairment to Alzheimer's disease"*. J Alzheimers Dis. 2010; 20(1): 97-104.

4. Lanni C, Racchi M, Uberti D, Mazzini G, **Stanga S**, Sinforiani E, Memo M, Govoni S. *"Pharmacogenetics and pharmagenomics, trends in normal and pathological aging studies: focus on p53"*. Curr Pharm Des. 2008; 14(26): 2665-71.

Papers published as result of other collaborative activities (2010)

During my PhD I also extended my interest on themes different from those described previously.

In particular, I studied the processing of the amyloid precursor protein (APP) focusing on a kinase named Casein Kinase 2 (CK2). We demonstrated for the first time that CK2 is a component of the complex cellular mechanism, activated following cholinergic stimulation, regulating the non-amyloidogenic processing of APP which conducts to an increased release of soluble fragments of APP (sAPPalpha), thought to have neuroprotective properties.

1. Lenzken SC, **Stanga S**, Lanni C, De Leonardis F, Govoni S, Racchi M. *"Recruitment of casein kinase 2 is involved in AbetaPP processing following cholinergic stimulation"*. J Alzheimers Dis. 2010; 20(4): 1133-41.

Moreover, I collaborated in writing a review about the action of neurotrophic factors (NTFs) in the response to multiple molecular, cellular, structural and functional changes that occur in the brain during aging, since they regulate the development, maintenance and survival of neurons, glia and oligodendrocytes and because a vast amount of evidence indicates that alterations in levels of NTFs or of their receptors can lead to neuronal death and contribute to aging.

2. Lanni C, **Stanga S**, Racchi M, Govoni S. *"The expanding universe of neurotrophic factors: therapeutic potential in aging and age-associated disorders".* Curr Pharm Des. 2010; 16(6): 698-717.