Platelet phosphorylated TDP- 43: An exploratory study for a peripheral surrogate biomarker development for Alzheimer's disease

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Running title: *Platelet TDP-43, a dynamic biomarker for Alzheimer's disease*

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Keywords: TDP-43, human platelet, Alzheimer's disease, hippocampus, surrogate marker

ABSTRACT

Alzheimer's disease (AD) is the sixth leading cause of death in the United States. The World Health Organization predicted that the world population with AD will rise to about 75 million by 2030 [1]. Therefore, AD and other forms of dementia create a non-curable disease population, and a socioeconomic burden in the world's societies. It is imperative to diagnose AD and other neurodegenerative diseases at their early stage. Consequently, it is important to develop a blood-based biomarker so that the remedial or disease-altering therapeutical interventions for AD patients would be available at the early stages of the disease. We have identified an easy, feasible, cost-effective, and less invasive assay method that measures platelet phosphorylated Transactive Response DNA Binding Protein 43 (pTDP-43), which may be a potential biomarker candidate for the neurodegenerative diseases. This protein recently gained an attention in the development of several neurodegenerative diseases (i.e., AD, ALS,and FTLD). We have identified an assay platform and generated some preliminary data that may suggest that the platelet TDP-43 levels were increased (<65%) in post-mortem AD brain regions and that similar trends were also observed in AD patient's platelets. In this study, we propose that the platelet phosphorylated form of TDP-43 could be used as a potential surrogate biomarker that is easy to measure, reproducible, sensitive, and cost effective for screening patients with some early clinical signs of AD and can be used to monitor disease prognosis.

INTRODUCTION

As the world population is getting older, the incidence of Alzheimer's disease (AD) is rising. More than 5 million Americans are living with the disease and this number is projected to rise to 13.8 million by 2050. Furthermore, only 1in 4 people with AD have been diagnosed [2]. The rising cost of health care for AD patients has a negative socioeconomic impact on the world society as well as being burden on caretakers. The early diagnosis of AD could be critical for starting an effective treatment with that of current options as well as designing new competent disease-modifying approaches. There is a great need to improve early detection in the course of neurodegenerative diseases such as Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), frontotemporal lobar disease (FTLD), and others so that the timely application of disease-specific treatments would be effective. Current diagnostic tests for AD rely on expensive brain imaging technology that is available only to a few patients [3], cognitive and psychiatric assessments, the collection of cerebrospinal fluid (CSF) samples which requires invasive and lumbar puncture that has a negative public perception in several countries [4]; however, none show high reliability and sensitivity, and diagnosis is confirmed by post-mortem pathological examination. Therefore, to identify biomarkers for AD as well as other neurodegenerative diseases is an urgent task. More specifically, the new biomarker should be sensitive, specific, reliable, affordable , readily available for rural areas, and involve a non-invasive sampling method. Such biomarkers are in great demand for the early stages of neurodegenerative diagnosis and making dementia screening a viable approach. We have focused on an AD-specific peripheral cellular biomarker in this study. There are several fluid based biomarker candidates for AD [5-8]; however, either the milieu of the diagnostic biomolecules or the measurement platforms for them have made many of these biomarkers unfavorable candidates. There is a new potential biomarker candidate for AD, **T**rans-**a**ctivation **r**esponse DNA/RNA binding protein (TARDP). Due to its 43 kDa size, TDP-43 acronym will be used throughout this paper.

Substantial research has been conducted to decipher the role of TDP-43 in different cellular events as well as its role(s) in neurodegenerative disease states [9-13]. TDP-43 is ubiquitously expressed in all nucleated cells [14]. Although TDP-43 is a nuclear protein [15] , it has the ability to shuttle in-and-out between nucleus and cytoplasm due to having nuclear localization and nuclear export sequences [16-19] . Cytosolic TDP-43 is not well described yet; however the phosphorylated derivatives of TDP-43 in neurodegenerative diseases may be responsible forming hyperphosphorylated aggregates and

could be considered as a signature biomolecule. There are 28 potential phosphorylation sites in TDP-43 protein shown in **Fig. 1A**. The majority of the phosphorylation occurs in serine-rich C-terminus. In neurodegenerative diseases such as FTDL, AD, and ALS, the tissue levels of TDP-43 are increased, mostly located in the cytosol, and consistently observed in inclusion bodies in neurons [15] rather than the nucleus. In Pick disease (PiD), the presence of TDP-43 inclusions suggests that TDP-43 accumulation and modification are an important component of PiD [20] Post-translationally modified TDP-43 aggregates are also observed in post-mortem brain tissue sections [14]. TDP-43 positiveinclusion bodies are becoming more detectable in about 75% of the brain tissues from AD patients [10, 21]. The level of TDP-43 in blood may also be elevated in AD patients, suggesting that TDP-43 may be considered as a potential surrogate biomarker in neurodegenerative diseases [22, 23] . TDP-43 is prone to phosphorylation and cleavage if it remains in the cytosol [24]. A recent study has provided some evidence that TDP-43 mislocalization was an early or pre-symptomatic event and was later associated with neurons [25] . These studies suggest that post-translationally modified (i.e. phosphorylated) TDP-43 may be viewed as a disease specific protein. To monitor disease relevant biomolecules in the brain is a challenge due to unfeasibility and invasiveness of taking repeated samples from brain and spinal cord tissues. Therefore, sampling blood splatelets may serve a feasible media where the aberrant TDP-43 is detectable. Platelets are anuclear blood cell fragments that are derived from megakaryocytes [26] and share the biochemical properties of neurons [27-29]. Accordingly, several investigators consider using platelets as a venue to study the pathogenesis of neurodegeneration. We chose platelets to identify and measure both total and phosphorylated TDP-43 protein species (i.e., monomers and oligomers) in AD because (i) they are easy to repeatedly obtain from the patients with minimal distress (ii) their life span is short (7-10 days) [30] which will reflect dynamic changes on phosphorylated TDP-43, (iii) it was reported that platelets transiently open the blood brain barrier (BBB) [31], consequently, biomolecules may come in to contact with the blood stream and become absorbed by platelets, (iv) serum/plasma proteins and other biomolecules are exposed to dilutions and results in analytical challenges, and (v) serum albumin and immunoglobulin interference for the assay are minimized.. In this study, we aimed to demonstrate that phosphorylated TDP-43 protein species can be specifically determined in AD patients' platelets and that the phosphorylation status of TDP-43 is AD specific as compared to another neurodegenerative disease such as ALS.

MATERIAL METHODS

Reagents*:* Anti hTARDBP polyclonal antibody (ProteinTech Group, Chicago, IL; Cat#1078-2-AP) and phosphorylated derivatives of the pTDP-43 antibodies (CosmoBio USA; Cat#TIP-TD-P09, TIP-TD-P07, TIP-PTD-P05, TIP-PTD-P03, TIP-PTD-M01, TIP-PTD-P01, TIP-PTD-P02, TIP-PTD-P04) (Abcam Cat# Ab184683 , ProteinTech Cat# 10782-2-AP,66318-1-Ig,22309-1-p(discontinued); Sigma Cat# T1705,SAB4200225; Biolegend Cat# 829901) were commercially purchased. Citrate Wash Buffer (11mM glucose, 128mM NaCl, 4.3 mM NAH_2PO_4, 7.5 mM Na_2HPO_4, 4.5 mM sodium citrate, and 2.4mM citric acid, pH 6.5)[32] and platelet rupture Buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) were prepared in our lab using reagent grade chemicals. Phosphatase inhibitor cocktail (Calbiochem # D00147804) (1:1,000) and protease inhibitor cocktail (Calbiochem# 539134) (1:2,000) were added to the platelet rupture buffer just before use to preserve TDP43 proteins from proteolytic degradation and dephosphorylation processes.

Human Platelets*:* Human blood-platelet samples were obtained from the following sources; (1) The Bio-specimen Bank of University of Kansas Medical Center (KUMC); platelets were previously collected from AD patients and age-matched with otherwise healthy subjects and stored at -80^{0}C and (2) ALS clinic at the University of Kansas Medical Center, Kansas City. ALS patient platelet lysates were utilized as a disease control for identifying a specific antibody for AD patient platelets. The ALS patients were clinically diagnosed by physicians and the subject identities for the biosamples were deidentified. All patients and otherwise healthy individuals were given a consent form before obtaining the blood samples. The sample collection procedure was approved by the Institutional Review Board of Kansas City University of Medicine and Biosciences (KCU) and the University of Kansas Medical Center (KUMC).

Platelets were isolated from freshly drawn blood from clinically diagnosed patients and otherwise healthy subjects according to a standard two-step low speed centrifugation technique as described in the literature with some minor modifications [33]. The platelet pellets were ruptured, sonicated in 0.6 ml of rupturing buffer with protease and phosphatase inhibitors, and subjected to high speed centrifugation (16,000 x g; 30 min; 4^{0}C) to obtain platelet cytosol. Protein concentrations were determined by the BCA spectrophotometric method [34]. The samples were aliquot and stored at -80^{0}C until use.

Human Brain Sample preparation

The brain tissue samples from post-mortem AD patients and age-matched control subjects were obtained from the Bio-specimen bank of KU Medical Center. The 100-200 mg samples of excised tissue from three brain regions (frontal cortex, cerebellum, and hippocampus) were removed and

homogenized in a Teflon-pestle glass homogenizer containing. ice-cold buffer (0.32 M sucrose, 0.5 mM $MgSO_4$, 10 mM epsilon-caproic acid, 0.1 mM EGTA, protease inhibitor cocktail 0.1% v/v, 10 mM HEPES, pH 7.4). Tissue : Homogenate buffer ratio was kept at 1:20. The homogenization was carried out in an ice bucket with 8-10 strokes. The homogenate was aliquoted and stored in -80^{0}C until use. Protein concentrations were analyzed by the BCA method [34].

Western Blot: The brain homogenate and platelet proteins were resolved in 12 % SDS-PAGE and 4-20% SDS-PAGE, respectively under the reducing conditions. The proteins were transferred onto a PVDF membrane and subsequently the membrane was probed with both pan anti-TDP-43 and several anti-phosphorylated TDP-43 antibodies. The protein bands were visualized by enhanced chemiluminescence and infrared dye based fluorescence methods, and they were analyzed by NIH's ImageJ (V.1.46r) and Image Studio^{TM} Lite software (V. 4.0)

Capillary Electrophoresis*:* The platelet lysates from AD, ALS patients and otherwise healthy subject cohort were analyzed by a simple western system, a new technology developed by ProteinSimple, Inc., USA. This technology does not require classical SDS/PAGE and Western blotting components. It uses very little sample mix volume (~3-5 ul). The samples were analyzed in duplicate and both capillary electropherogram and pseudo protein bands were generated and analyzed by the system software (Compass for Simple Western, v.3.0.9).

Statistical analysis: Paired t-test was employed for statistical analysis.

RESULTS:

TDP-43 protein levels differentially increase in AD-patient brain tissue and this increase is reflected in platelets. In the early stages of this work, we have shown that total TDP-43 protein levels were increased in the brain regions of post-mortem AD patients (n=3). The most noticeable TDP-43 increase was observed in the hippocampus while the frontal cortex and cerebellum reflected a slight TDP-43 increase as compare to non-symptomatic control subjects (**Fig. 2A**). Total TDP-43 aggregates were observed in three different brain regions and the most notable aggregates were observed in the hippocampus (**Fig.2B**). We have also observed that the platelet lysate TDP-43 levels were increased by <65 % in AD patients (n=3) (**Fig. 2C**) in the early phase of this study. Readers should be advised that platelet lysates were obtained from a separate AD patient cohort, because the University of Kansas Medical Center Bio-specimen repository did not have the matching post-mortem tissue and platelet lysates from the same AD patients and non-symptomatic control individuals.

A sequence specific anti-phosphorylated TDP-43 Ab distinguishes AD from other neurodegenerative disease. In the next phase of this work, we have focused on identifying an AD specific anti-phosphorylated TDP-43 Ab as a screening tool in a relatively large subject cohort (n= 10 in each group). First, we employed a computer based **P**redictor **o**f **N**atural **D**isordered **R**egion (PONDR®) algorithm using TDP-43 sequence (NCBI accession code: Q5R5W2.1). Disordered Enhanced Phosphorylation Predictor (DEPP) analysis predicted 28 potential phosphorylation sites and a majority of them were Ser amino acid enriched on the C-terminus (aa 369-410) (**Fig.1A**). Another algorithm (PONDR® VL3-BA) was employed to predict 152 aa long regions of disorder that were characterized by other methods (**Fig.1B**). Nuclear magnetic resonance (NMR) studies also revealed that an ~ 80 aa sequence from the C-terminus region of TDP-43 was identified as the most disorderly region [35, 36] where the majority of phosphorylation sites were located. Therefore, we have tested several anti- Phosphorylated TDP-43 antibodies from various vendors (ProteinTech, Abcam, Cosmobio-USA, Sigma, and Biolegend) to identify an AD-specific antibody that can be used for screening assays. An anti-phospho (S409/410) TDP-43 antibody (ProteinTech Cat# 22309-1-AP) was identified as a potential antibody that discriminates AD platelet lysate phospho-TDP-43 profile from that of amyotrophic lateral sclerosis (ALS) (**Fig. 3A**) and from that of non-symptomatic, otherwise healthy age-matched subjects (**Fig.3B**). A prominent protein peak at about 62 kDa position was consistently observed in platelet lysates (**Fig.3A**).

DISCUSSION:

Misfolded aberrant protein aggregations are frequently observed in neurodegenerative diseases [37]. Pathologically misfolded protein aggregate formation occurs long before any measurable cognitive decline [38]. Therefore, it is essential to develop a feasible, cost-effective, and specific method or an assay system to analyze the biomarker biomolecules. This test may aid medical evaluations to predict AD before the clinical manifestations are revealed.

Intracellular TDP-43 species such as aggregates, cleaved TDP-43 fragments, and post-translationally modified TDP-43 have been found in neurodegenerative diseases [39]. The characteristics of TDP43 as a regulator of mRNA translation and an inducer for stress granule (SG) formation may suggest that post-translationally modified TDP-43 may affect the pathological course of the diseases much earlier than previously thought [40] . A cell-based TDP-43 chemical modification and aggregation model may be a good strategy [41] to investigate whether peripheral cells would be considered as a platform

where the surrogate biomarker such as TDP-43 can be analyzed. Therefore, we have hypothesized that platelet phosphorylated TDP-43 may be considered as a viable surrogate dynamic biomarker.

In this study, we have provided some new findings that platelet TDP-43 and it's phosphorylated derivatives may reflect the changes in the TDP-43 profile in human AD brain. We have focused on platelets for several reasons: (**i**) the life span of circulating platelets is about 8-10 days [42]. The half-lives of TDP-43 was studied in primary fibroblasts obtained from human ALS patients that have dominant G298S mutation in TDP-43 [43]; the half-life of mutated TDP-43 ($t_{1/2}$ = ~11 hours) was extended by about 2.8-fold over the wild-type cells ($t_{1/2}$= 14 hours). Although no half-life studies on platelet TDP-43 was conducted, platelet TDP-43 may reflect the current profile of aberrant TDP-43; (**ii**) platelets secrete platelet activating factor which induces transient blood brain barrier (BBB) opening [31] where aberrant TDP-43 loaded glia cells may come in to contact with blood cells and TDP-43 would be transferred via cell-to-cell contact; (**iii**) platelets are anuclear blood cell fragments originated from megakaryocytes and reflects mostly cytosolic TDP-43, which are more prone to modification (i.e., phosphorylation, aggregation, and fragmentation), and (**iv**) platelets are very easy to obtain from venous blood with a minimum invasiveness for patients' comfort, and (**v**) repeated sampling is possible to study the progress of disease. These are the known advantages of platelets as a platform to analyze theTDP-43 protein profile which reflects similar changes in the central nervous system.

To identify an AD-selective antibody was a major undertaking. We have tested several antibodies (eight) that raised against to different regions of TDP-43 as well as phosphorylated species of TDP-43 that were purchased from three vendors (ProteinTech, Cosmobio-USA, Abcam). Among these, we have identified an anti-phospho (S409/410)TDP-43 antibody from ProteinTech as an AD-selective antibody. Although, several other antibodies that were raised against to same region (i.e. S409-410) of TDP-43, ProteinTech antibody was shown to be selective for AD samples. It may be due to either the antibody producing clone is different or TDP-43 modification is different in AD than ALS. We have used ALS platelet samples for testing the specificity of the antibody that showed high levels of pTDP-43. We are now identifying an ALS-selective antibody that does not show positive reaction for AD platelet lysates in a separate project.

There are several studies in the literature that have reported TDP-43 levels in serum and brain samples obtained from AD patients. Kadokura et al, reported that more than 30 % of diagnosed AD cases showed TDP-43 pathology [44]. Similar studies were also reported elsewhere [10, 45, 46]. All of these studies provide considerable

supporting evidence that a notable percent of AD cases are linked to altered TDP-43. Foulds et al., have suggested but have not definitely showed that plasma TDP-43 levels might discriminate AD with TDP-43 pathology from those without TDP-43 pathology [22]. We think that their inconclusive observation may be due to the complex nature of serum which does not reflect the chemical modifications of TDP-43 based on the ELISA method. It should be considered that unless the primary antibody used in ELISA is an isoformic specific for the target protein, the method will not provide target protein specific data. Serum contains some very abundant biomolecules such as albumin and immunoglobulins. These biomolecules may mask the levels of TDP-43 in serum based assays so that positive recognition of TDP-43 by it's specific antibody may be greatly reduced. That is why we justified turning to platelets as a biological milieu, which will reflect a more concentrated and encapsulated population of TDP-43 without interference of serum albumin and immunoglobulins.

Herman et al., have observed an increased level of TDP-43 in cortical autopsies of AD patients [47], suggesting that TDP-43 pathology may be the common point among AD, ALS, and Frontotemporal lobar dementia (FTLD). We also believe that TDP-43 is situated in a very critical position of several neurodegenerative diseases. Youman and Wolozin further placed TDP-43 as a causative factor in AD since TDP-43 has been shown to increase A-β accumulation through increased β-secretase activation [48]. It is not clear whether normal TDP-43, cleaved, and/or post-translationally modified TDP-43 activates β-secretase. Herman et al., have demonstrated that $A\beta_{1-42}$ increases the full length, cleaved, and phosphorylated TDP-43 levels, which in turn further increases the β-secretase activity which will produce more $A\beta_{1-40}$ and APP C-terminal fragments [49]. This observation is critical in the involvement of TDP-43 in AD progression. However, a recent study puts more emphasis on extreme N-terminus modification of TDP-43 showing that such modification activates caspase-3 [50] and subsequently the cleavage of TDP-43 proteins since TDP-43 has three caspase cleavage sites (Entrez accession NP_031401) that generate ~ 42,35,and 25 kDa TDP-43 fragments; however, the fragmentwhich is more fibrillogenic remains unknown [51]. A recent study has reported a new caspase-4 cleavage site at Asp174 that produces ~25 kDa C-terminal fragment [52]. In another study, the investigators have shown that a mammalian enzyme asparaginyl endopeptidase cleaved and produced two immunogenic TDP-43 fragments (35 and 32 kDa) [53]. These fragmented TDP-43 species are more likely encapsulated in immunoreactive inclusion bodies that may be associated TDP-43 relevant disorders [51]. In our view, there are several enzymatic cleavages of TDP-43 that produces cleaved toxic TDP-43 fragments that

may be easily phosphorylated. Subsequently, these fragments will first form an aggregation nucleus through protein-protein interactions yielding TDP-43 enriched plaques in CNS tissue. All of these cited studies as well as many others strengthened the conception that TDP-43 protein profile in Alzheimer's disease may be a good dynamic biomarker that ought to be comprehensively studied.

TDP-43 proteinopathy is characterized by decreased solubility, hyper-phosphorylation and the generation of 25kDa C-terminal fragment [15, 54-56]. We also have observed ~35 and ~25 kDa TDP-43 fragments in early stage of this work; we thought that they may represent the degradation products of TDP-43 due to either storage of samples at -80^{0}C for extended period of time or the degradation is due to the old age of the subjects (**Fig. 2C**). This observation leads to future studies that ought to be conducted that address the TDP-43 fragmentation issue. In addition to these findings, we have also noticed TDP-43 protein aggregation in select brain regions (**Fig.2A, 2B**). We anticipated the observation that hippocampal TDP-43 protein aggregation levels would be relatively high and statistically significant ($P \leq 0.015$; t-test) (**Fig. 2A**). We have shown in our previous studies that the hippocampal region is very vulnerable to oxidative stress in the aging process [57]. This also partially explains that increased levels of TDP-43 aggregation in the hippocampus region.

In tissue, cytosolic TDP-43 protein , especially toxic monomers [58], begin to form hyperphosphorylated species which are sequestered into inclusion bodies as part of the defense mechanism of the organism, suggesting that cytosolic pTDP-43 or detergent-soluble TDP-43 protein is toxic [9]. We did not verify inclusion body presence in platelets. What we know is that cytosolic TDP-43 is present in platelets and phosphorylated species of TDP-43 are elevated in Alzheimer's disease. We speculate that anuclear platelet cytosol represents the toxic form of TDP-43 species. How does aberrant brain TDP-43 appear in peripheral blood cells? One explanation might be that the TDP-43 protein has a C-terminus Q/N rich region [59]; therefore, this protein may have the characteristics of prion-like proteins that propagates itself [60, 61] and transfects other cells. Kanouchi et al., have reviewed the recent findings about the prion-like characteristics of TDP-43 propagation and offered the concepts of contiguous and non-contiguous propagation of misfolded proteins including TDP-43 [62]. Considering the leaky BBB in neurodegenerative diseases as well as the ability of platelets to transiently open the BBB via releasing platelet activating factors [31], it is conceivable that aberrant TDP-43 in astrocytes may transfect the blood cells by means of cell-to-cell infection through having access to the blood stream. Conversely, one can argue that platelets are the pTDP43 carrier as part of AD

development and load the glial cells by cell-to-cell infection through a leaky BBB. The concept of cell-to-cell misfolded protein infection was recently reviewed [63]. Our present data only suggests that the observed elevated TDP-43 protein pattern in AD brain was reflected to the AD patient's platelet TDP-43. Yet, we are well aware that we were unable to obtain the platelets and post-mortem brain tissues from the same subject, which could be the better representation of the TDP-43 profile. In this study, we have provided a trend of TDP-43 profiles in AD and age-matched healthy subjects (**Fig. 2A, 2C**). We are in the process of searching nation-wide biorepositories to obtain platelets and post-mortem brain samples from the same individuals and we will repeat our assays to verify the results presented in this study. To our knowledge, we are the first research group to identify the TDP-43 profile in platelets which could be considered as a surrogate dynamic biomarker to monitor the disease progress as well as the pharmacological treatment response.

Our findings about the presence of phosphorylated TDP-43 in platelets from AD patients are intriguing and led us to question whether AD is an exclusively CNS or peripheral system disease? This issue is currently being studied and requires some very comprehensive studies [64] [65].

The other provocative hypothesisiwould be that mitochondria may be a potential target for the soluble TDP-43 protein and it's fragmented derivatives (i.e., ~35, ~25 kDa fragments). The malfunction of mitochondria and low levels of bioenergetics are hallmarks in neurodegenerative diseases and this issue had been discussed elsewhere [66, 67]. We have observed a ~ 25 kDa TDP-43 species in mitochondria-enriched preparations from healthy human platelets. (Supplemental **Fig.1**). We don't have an answer to whether this is mislocalization of TDP-43 fragments or naturally occurring in mitochondria. However, we may consider to decipher the relationship between TDP-43 and transport protein of outer membrane (TSPO) of mitochondria [68], which may explain how TDP-43 fragment entered into mitochondria.

We have had some obstacles to obtaining a sufficient number of control and ALS post-mortem human brain and spinal cord tissues to correlate with the platelet TDP-43 levels due to limited availability of such samples in local biorepository to provide supporting data that this notion would be true in other neurodegenerative diseases. The challenging question would be when does TDP-43 begin to form aggregates? Which TDP-43 species initiate the seed for inclusions? It is well known fact that protein aggregations occurs long before the clinical manifestations are revealed [37] . In vitro biophysical studies in cell culture and mouse brain have suggested that TDP-43 naturally tends to form a dimeric protein as cited in a recent review [69]. Can we monitor

TDP-43 modifications and aggregations during disease progression? This issue was always a challenge and led us to plan a longitudinal studies in future. Perhaps the platelet TDP-43 approach will make these kinds of studies feasible. As discussed by Budini et al., cell-based TDP-43 aggregation and modifications model is a powerful tool [41] to test novel therapeutic strategies aimed at preventing and/or reducing TDP-43 aggregation in AD.

In the near future, as suggested by Cohen and Kelly [70], researchers may consider some therapeutic approaches by which cell permeable chemical chaperons that bind to misfolded protein and stabilize the folded state reduce protein misfolding. In normal circumstances, the molecular chaperons and other housekeeping mechanisms ensure that potentially toxic aberrant proteins or pre-fibrillary aggregates are neutralized before they can do cellular damage [71, 72]. Therefore, the researchers need to know the folding features of protein of interest. If we know the folding features of TDP-43 and can measure the occurrence of misfolded, disease prone TDP-43 early enough, we may be able to stabilize the misfolded protein, which opens up new therapeutical venues for neurodegenerative disease treatment.

Acknowledgements

Research reported in this publication was supported by several pilot project funds from QS85523J, University of Kansas Medical Center Research Institute, Inc.(QS85523J), FONTIERS-Trail Blazer Award (01-2429-001), and KCU intramural grants. AA acknowledges the contributions of the student research fellows of College of Osteopathic Medicine. We are grateful for Drs. Eric Vidoni and Kathy Newman, and KU Medical Center biorepository facilities for providing brain tissues and platelet lysates. We are thankful for Emre Agbas for editing process of this manuscript.

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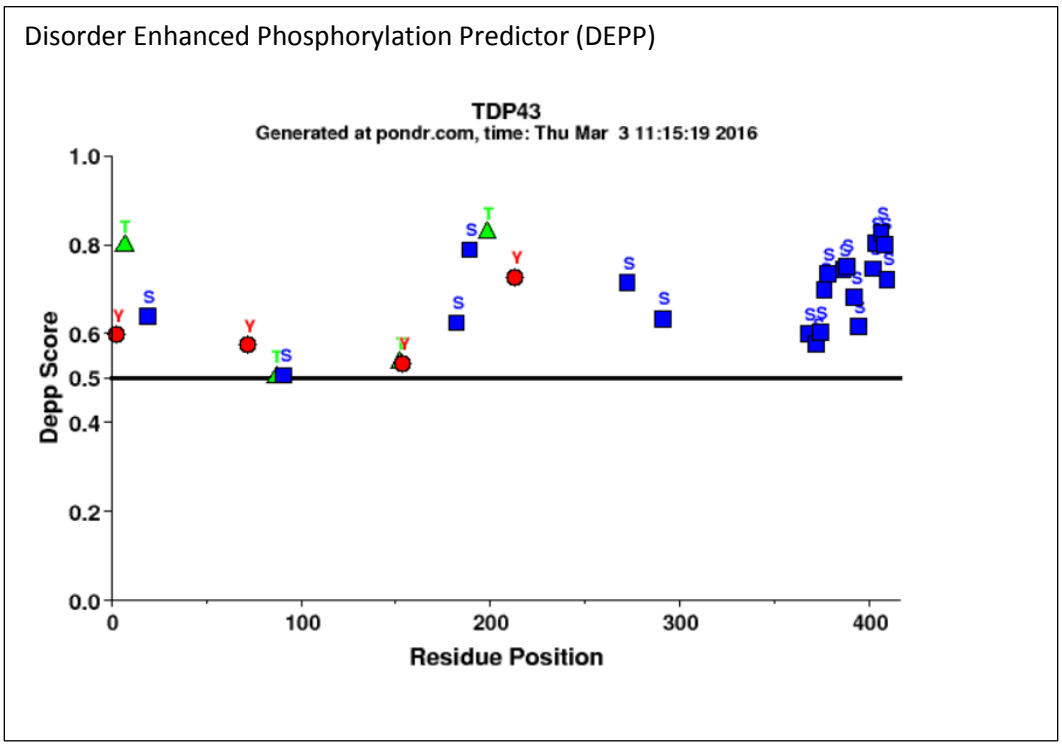

Fig. 1A. PONDR ® analysis of TDP43 for potential phosphorylation sites. Majority of the phosphorylation events were predicted at Serine (Ser) amino acid sites (359-410). Most of the Ser amino acids are located at C-terminus region (20 out of 41; 48.7%)

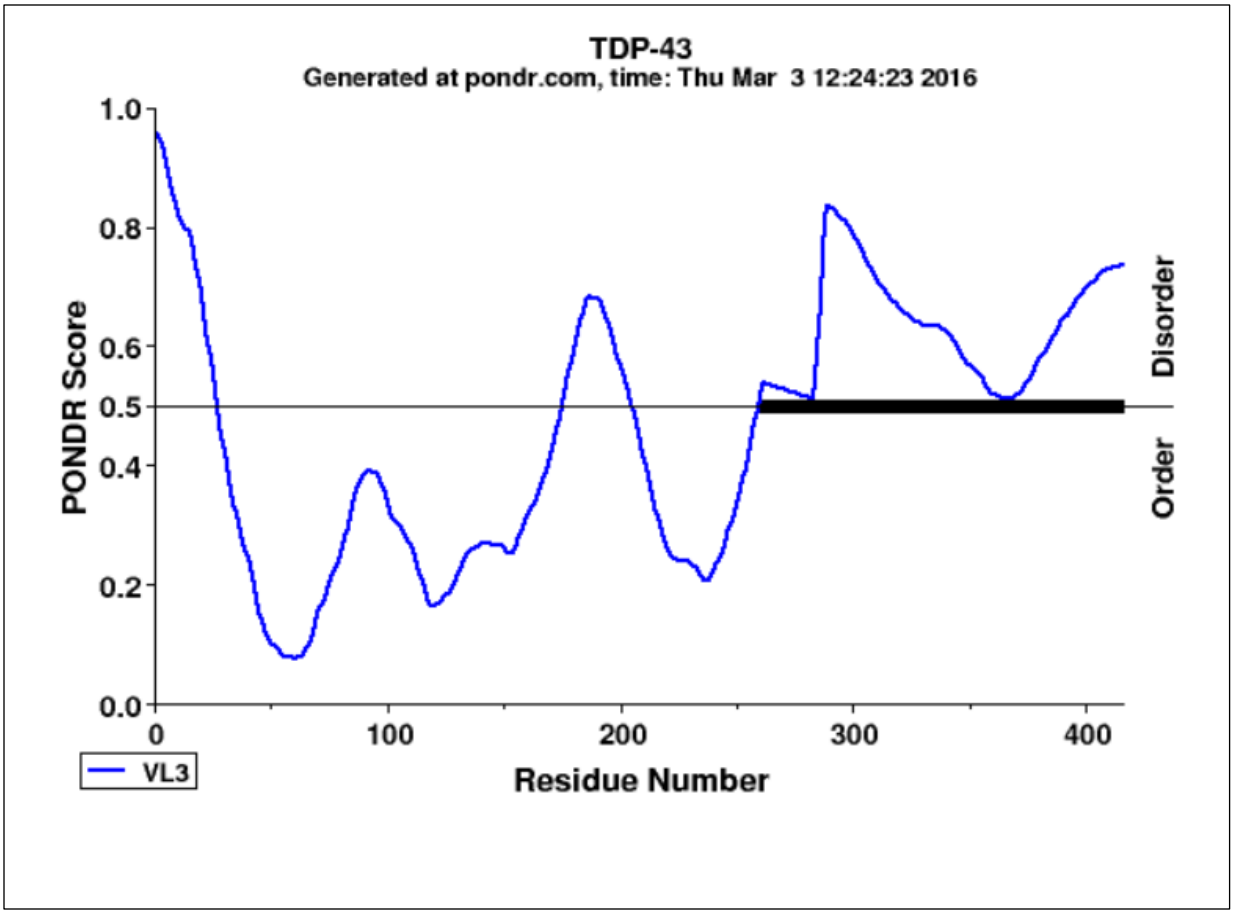

Fig.1B. PONDR ® VL3-BA analysis of TDP43 for identifying the disordered sites.

The VL3-BA predictor is a feedforward neural network that was trained on regions of 152 long regions of disorder that were characterized by various methods. The region close to C-terminus was identified as disordered sites for TDP-43 which is also most of the Serine amino acids are located.

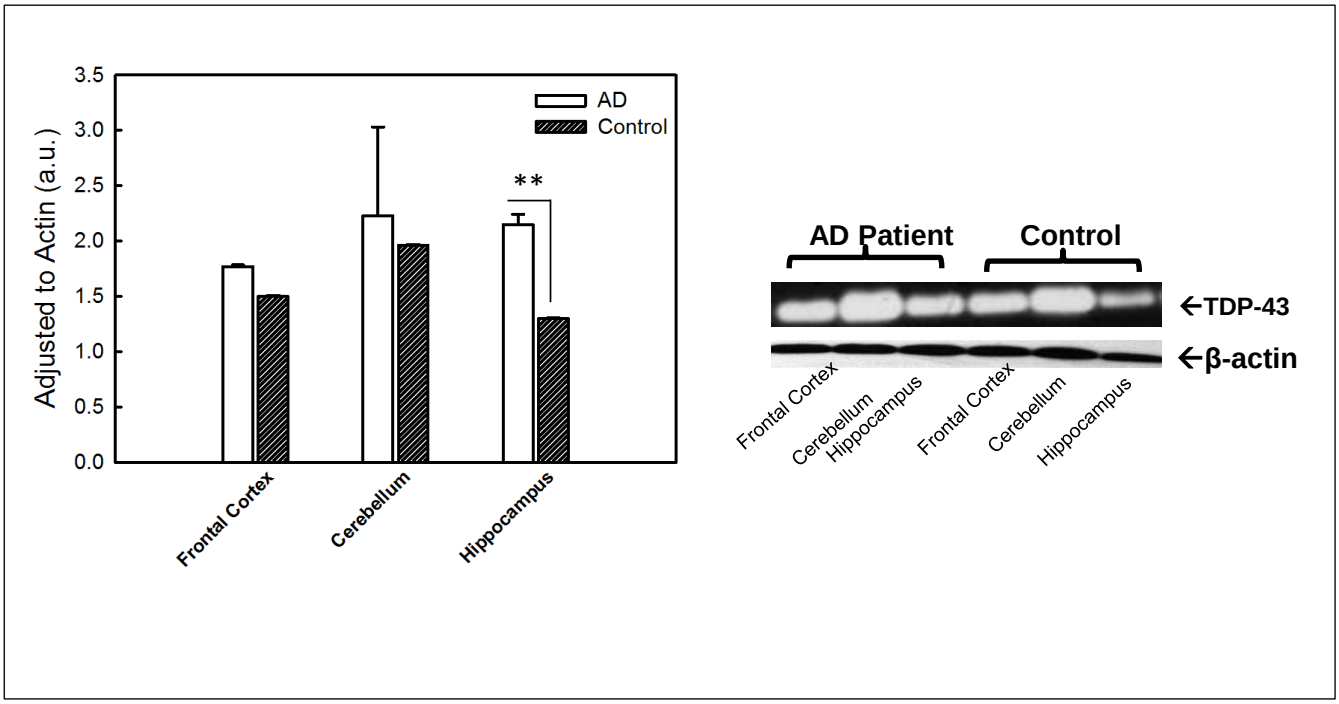

Fig. 2A. TDP-43 distribution in Alzheimer's disease (AD) patient brain regions. The tissue from three different regions of the brain was used in this study. The tissue homogenates were analyzed by immunoblotting method. The protein band intensities were normalized to actin. Three post-mortem AD patients and age-matched healthy human brain samples were utilized in this study (n=3). The difference between control and AD in hippocampus region was found statistically significant (P≤0.015) according to paired t-test.

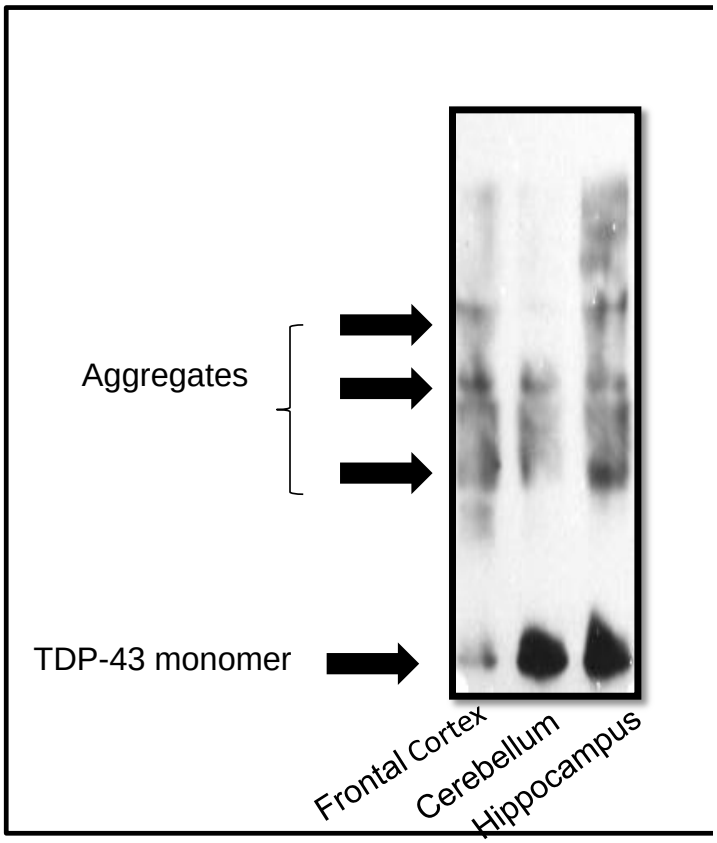

Fig.2B.TDP-43 protein aggregation in Alzheimer's disease patient's brain region.

The homogenates from different regions of the brain were resolved in non-reducing SDS/PAGE condition and immunoprobed with anti-TDP-43 (pan) antibody (1:1000 dilution). The TDP-43 protein aggregation is relatively more prominent in hippocampus region.

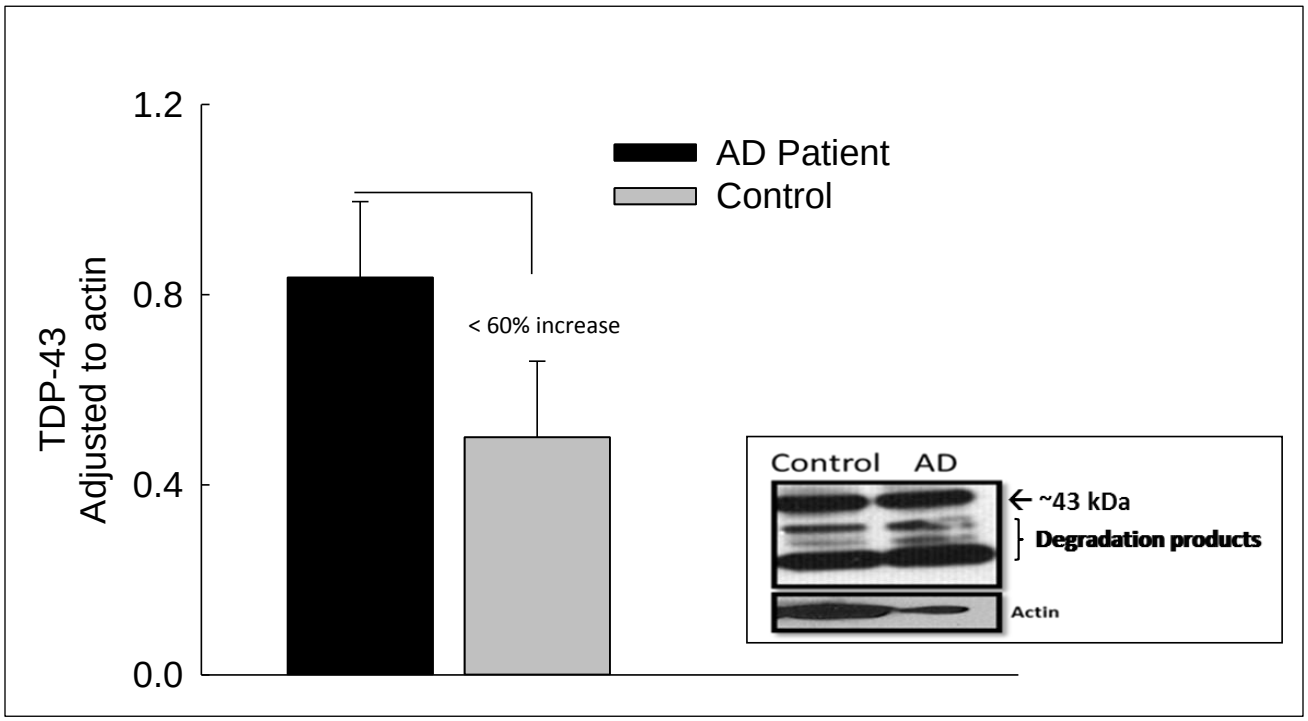

Fig. 2C Platelet lysate TDP-43 profile. This figure represents the early TDP-43 studies on platelets obtained from AD patients (n=3) and age-matched healthy subjects (n=3). The platelet lysates were analyzed by a classical immunoblotting assay using anti-TDP-43 (pan0 antibody (1:1000 dilution).

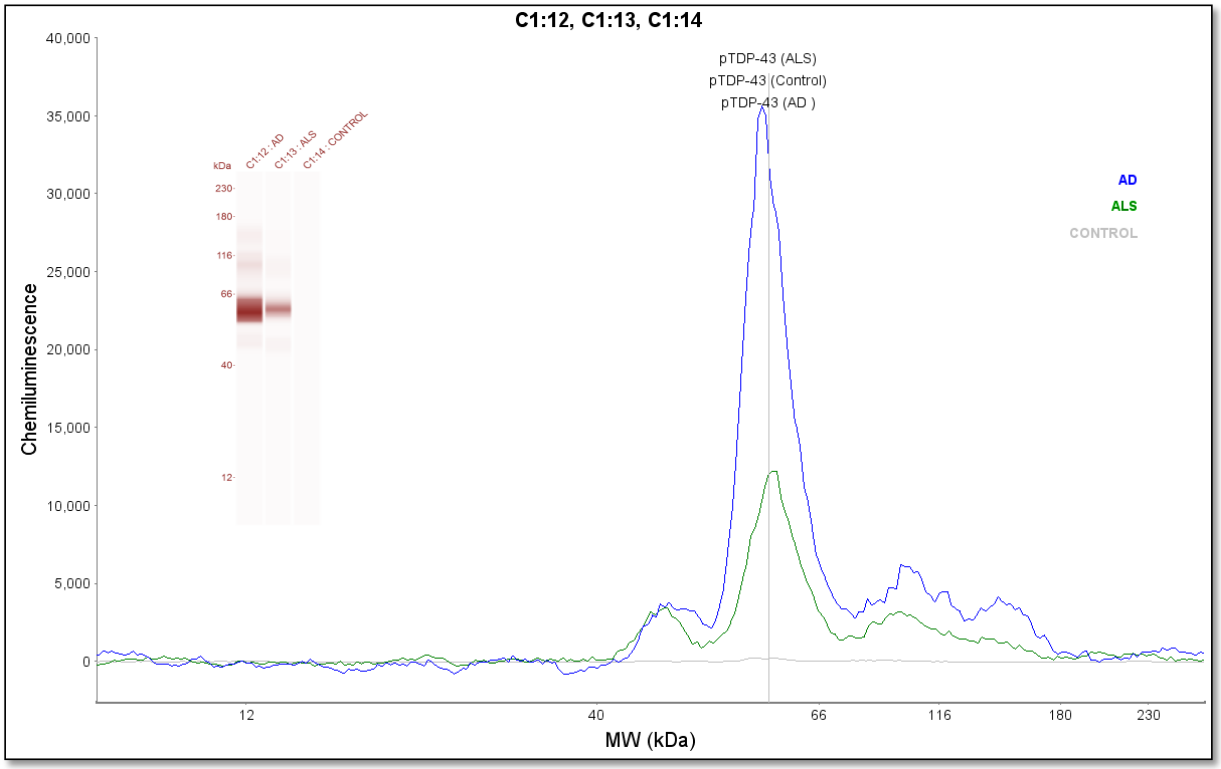

Fig. 3A Phosphorylated TDP-43 profile in platelet lysates.

An anti-phospho (Ser409/410-2) TDP-43 antibody was used as an immunoprobing agent. The signals from AD platelet lysates was more pronounced as compare to ALS (disease control) and healthy subjects (control). Inset figure shows a computer generated pseudo band that marks prominent phosphorylated TDP-43 at about 62 kDa.

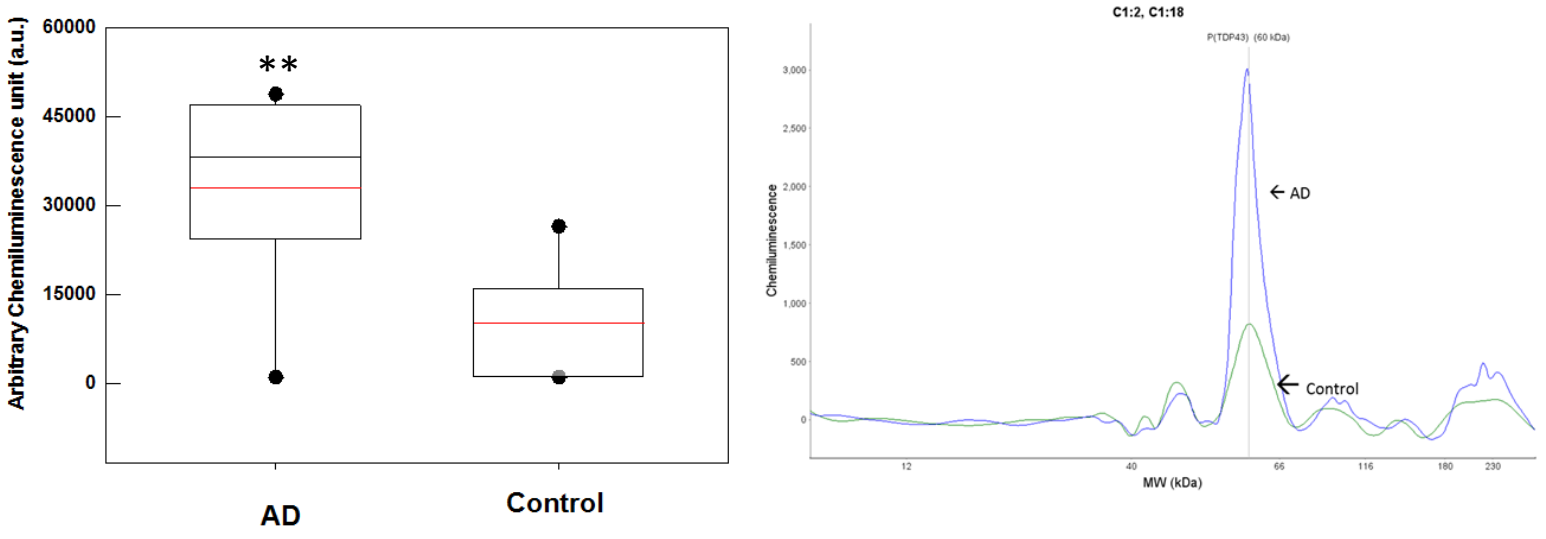

Fig. 3B. Platelet lysate phosphorylated TDP-43 protein profile. The platelet lysates obtained from biospecimen bank (n=10 in each group) and analyzed by capillary electrophoresis based gel-less and membrane-less system western assay developed by Proteinsimple (WES). The electropherogram peaks indicate the noticeable difference at about 62 kDa protein that represents phosphorylated TDP-43 protein. Box-whiskers plot represents statistical values. Redline within the boxes mark the median. A paired t-test was employed for statistical analysis. Difference between AD and control platelet phosphorylated TDP-43 was found statistically significant ($P \leq 0.010$)