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INTERACTING CYTOSKELETAL AND METABOLIC PROTEINS: ROLE IN THE DEVELOPMENT OF ALZHEIMER'S DISEASE

Beena Hasan[1,2], Ayesha Khan[1], Abid Azhar[2] and Nikhat Ahmed[*1,3]

[1]*Neurochemistry Research Laboratory, Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan.*
[2]*The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi-75270, Pakistan.*
[3]*Department of Biochemistry, Barrett Hodgson University, Korangi Creek, Karachi-74900, Pakistan.*
[*]**Address correspondence and reprints request to:**
Email: nikhat_ahmed14@yahoo.co.uk; nikhat.ahmed@bhu.edu.pk

ABSTRACT

Alzheimer's disease (AD) is a complex neurodegenerative disorder of impaired proteostasis. An in-depth understanding of protein complexes, their components, interaction and post translational modification (PTM) is crucial for gaining insight of AD pathology. This study aims to identify novel disease associated protein interactions and their potential phosphorylation and glycosylation (*O*-GlcNAc) involved in the progression of AD, to help unravel the underlying disease mechanisms.

Thirteen protein complexes were obtained on Blue native PAGE (BN-PAGE) from human brain prefrontal cortex of AD and age matched controls. Complex VII (305Kda) entailing pronounced alteration was further resolved into its components on SDS-PAGE and identified by mass spectrometric analysis. The differentially expressed proteins were mapped to existing biological networks and analyzed for potential phosphorylation and *O*-GlcNAc. Glyceraldehyde-3-PO_4 dehydrogenase (GAPDH), actin cytoplasmic (ACTB), microtubule associated protein 1B (MAP1B), myelin proteolipid protein (PLP1), acyl amino acid releasing enzyme (APEH) were found to be differentially expressed among AD and control brain. Further, network analysis reveals a strong interaction between metabolic proteins (GAPDH, APEH) and their cytoskeletal counterparts (MAP1B, ACTB, PLP1).

The annotated network and pathways associated with altered proteins along with their PTM warrants further research to study their actual contribution in AD pathology.

Keywords: Alzheimer's disease, Cortex, Protein complexes, BN-PAGE, GAPDH, ACTB.

INTRODUCTION

Alzheimer's, a complex disease of progressive neurodegeneration is characterized by multiple alterations in protein interactions (Talwar *et al*., 2014). Protein-protein interactions (PPIs), either stable or transient, intrinsic to virtually all cellular processes, can be well elucidated by proteomic tools and applications (Klapa *et al*., 2013). With current developments in the fields of proteomics and system biology, the interest in deciphering protein complexes and the interaction among multiple protein components with an overall impact on the cellular functioning and disease pathophysiology has greatly enhanced our understanding of AD progression (Malhotra *et al*., 2015).

Although, proteins accomplish their biological function in association with other proteins and seldom work in isolation, several non-covalent aberrant interactions among protein components results in a disease state. Potential biological role of a protein in orchestrated cellular environment can be inferred from its protein-protein interaction network (Hayes *et al*., 2016) which can be altered by the conformational state of protein or its post translational modification (PTM). Disease associated genes and proteins and their relationships can be well studied from the network biology of data generated from human protein-protein interactions (PPIs) (Lee *et al*., 2011).

Molecular events of learning and memory, associated with PTMs alter the synaptic plasticity affecting the cognitive processes. Phosphorylation and *O*-GlcNAc of proteins at their Ser/Thr residues is a dynamic modification regulating protein folding, processing, distribution, trafficking and interactions. Many attachment sites of kinases and *O*-GlcNAc transferase are identical. These sites are known as Yin Yang sites for their property to alternatively carry a phosphate or carbohydrate residue at the same or neighboring Ser/Thr residues. This interplay has a role in signaling cascades (Kaleem *et al*., 2009). Altered protein phosphorylation is a defining feature of AD. Multiple proteins exhibit dysregulated functions in AD due to aberrant phosphorylation (Zahid *et al*., 2012). Likewise altered protein glycosylation has also been observed in AD and postulated to play a key role in the pathogenesis (Frenkel-Pinter *et al*., 2017).

Due to lack of data on protein interactions, their PTM and implications on neurodegenerative diseases, this study was undertaken. The present work explored the interacting components of a protein complex, their interaction pattern, expression aberrations and PTM in AD and age-matched control brain prefrontal cortex. Our analysis underscores pivotal contribution of aberrantly expressed proteins in various molecular and biological processes chiefly cytoskeletal assembly and metabolic pathways. In the past, interactive relation of cytoskeletal proteins and metabolic enzymes has not been focused in AD. TP53 a major contributor in neurodegenerative disorders (Chang *et al*., 2012) has been found to interact with the differentially expressed proteins, identified by network analysis, thus implicating a mechanism involving TP53 initiating apoptosis with integrated cytoskeletal and metabolic proteins. Our results revealed clear interaction pattern of structural and metabolic protein components of the cell depicting the role of metabolic enzymes in cytoskeleton stability, providing a better understanding of AD pathology.

MATERIALS AND METHODS

Human brain tissue

Brain frontal cortex autopsied samples from pathologically confirmed well characterized AD patients (n = 5) along with age matched unaffected control (n = 6) were obtained from The University of Edinburgh, (MRC Sudden Death Brain Bank), UK. The mean age of all the subjects was between 40-80 years. The tissues after excision were stored at -80^{o}C until further processed. The study has been designed in accordance with the guidelines for the use of human samples defined by ethical review board of University of Karachi, Karachi, Pakistan. All the experiments were run in triplicates to ensure reproducibility.

Solubilization of protein complexes

The isolation of protein complexes from the human brain prefrontal cortical region was performed according to the method of Wittig *et al*. (Wittig *et al*., 2006) with slight modifications. Briefly, the brain prefrontal cortex tissue (50mg) from AD and age-matched control was homogenized in sucrose buffer (250mM sucrose, 20mM imidazole/HCl, pH 7) using pellet pestle motor hand homogenizer (10-20 strokes). The homogenized samples were centrifuged at 20,000g for 10 min to obtain nuclei, mitochondria and large cell fragments in the pellet. The pellet was further homogenized in solubilization buffer A (NaCl 50mM, Imidazole 50mM, 6-aminohexanoic acid 2mM, EDTA 1mM, pH 7). The protein complexes were then solubilized in 20% dodecyl-maltoside for 30min with subsequent centrifugation at 100,000g for 15min. 50% glycerol and 5% Coomassie Brilliant Blue G-250 (CBB G-250) were finally added to the centrifuged supernatant. Triplicates of each sample were used to ensure reproducibility.

2D BN/SDS-PAGE and staining

Blue-native PAGE was run according to Wittig *et al*. (Wittig *et al*., 2006). Briefly, the protein complexes were separated on 4−16% blue-native gradient separation gel with a stacking gel of 3.5%. Electrophoresis was carried out at 100 V (4 °C) with the cathode buffer (7.5 mM Imidazole, 50 mM Tricine) containing 0.02% (w/v) CBB G-250 and the anode buffer (25 mM Imidazole, pH 7.0). It was continued at 200V with 0.002% of CBB G-250 containing cathode buffer. BN gel was fixed (50% methanol, 10% acetic acid and 100 mM ammonium acetate) followed by CBB G-250 staining. While the first-dimension electrophoresis was in progress, a second-dimension gradient SDS-PAGE gel (7.5% - 12.5%) was prepared and poured in mini gel plates (Bio-Rad, 1.5 mm spacers) leaving a gap of 1cm above the gel. From the first-dimension gel lane strips were cut and then slid orthogonally on of the second-dimension prepared gel using sample gel (3.5%) to seal the two gels. Single tooth of the comb was also adjusted during gel polymerization for marker. In second-dimension run was performed, initially at 100 V while in the sample gel and later continued in gradient gel at 15 mA for approximately 3 h. Proteins were visualized using standard CBB G-250 staining protocols (Schägger, 2006). The spots were excised and nano LC-MS/MS analysis was carried out. Reproducibility was maintained by running five gels of each sample.

Image and statistical analysis

Densitometric analysis of protein complexes on BN gel images was carried out by Quantity One software (BioRad). Whereas, the SDS-PAGE resolved protein components were analyzed by Melanie 7.0 (GeneBio) where in, a single gel is considered as a reference image having maximum number of spots. The spots were marked in the reference image and compared with all representative 6 images. The normalization was carried according to the total valid spot density in the gel. A twofold increase or decrease with a significance of $p < 0.05$ calculated by Student's *t*-test was considered as threshold for aberrantly expressed proteins. Spots obtained from brain frontal cortex of 5

AD patients were compared with 6 age matched control brain frontal cortex protein spots. Triplicates of each patient sample were run to achieve reproducible results.

Tryptic digestion and protein identification by Nano LC-MS/MS analysis

Protein spots of one complex out of thirteen containing highest number of interacting protein partners with differential expression were excised from the gels and analyzed by high resolution orbitrap mass spectrometry. For in-gel trypsinization gel slices were destained with a mixture of 15 mM $K_3Fe(CN)_6$ and 50 mM $Na_2S_2O_3$ and then dehydrated with acetonitrile (ACN), after being dried they were incubated with 100 mM NH_4HCO_3. ACN again poured, dried with vaccum and cleaved with sequencing grade, modified trypsin and incubated at 37^oC overnight. With moderate sonication, tryptic peptides were extracted twice with ACN (50%) and trifluoroacetic acid (TFA) (0.1%). The solutions extracted were pooled, again dried with vaccum and re-dissolved in 0.1% TFA. The samples were separated with reversed phase-C18 column (0.075 mm ID x 200 mm, Reprosil-Pur 120 C18-AQ, 3 µm) after enrichment on a self-packed reversed phase-C18 precolumn (0.15 mm ID x 20 mm, Reprosil-Pur120 C18-AQ 5 µm) and 15 min linear gradient of 5-35 % acetonitrile/0.1% formic acid (v:v) at 300 nl min^{-1}. The eluent obtained was investigated using Q Exactive hybrid quadrupole/orbitrap mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany) with a FlexIon nanoSpray source and operated under Excalibur 2.4 software. The experimental run was of one full MS scan across the 350-1600 *m/z* range acquired at a resolution setting of 70,000 Full-Width at Half-Maximum (FWHM), an Automatic Gain Control (AGC) target of 1*10e6 and a maximum fill time of 60 ms. Up to the 10 most abundant peptide precursors of charge states 2 to 5 above a 2*10e4 intensity threshold were then sequentially isolated at 2.0 FWHM isolation width, fragmented with nitrogen at a normalized collision energy setting of 25%, and the resulting product ion spectra recorded at a resolution setting of 35,000 FWHM, an AGC target of 2*10e5 and a maximum fill time of 120 ms. Selected precursor *m/z* values were then excluded for the subsequent 6 s (Atanassov and Urlaub, 2013).

Data processing

MASCOT software version 2.4 (Matrscience, London, United Kingdom) was used for identification of proteins. The seven proteins of the complex were identified against the UniProtKB with *Homo sapiens* species filter. The database was searched using enzyme trypsin and blocking agent iodoacetamide as cysteine. Methionine oxidation was set as a variable modification whereas up to two missed tryptic cleavages were allowed. ESI-QUAD-TOF was selected as the instrument type whereas the search tolerances were limit to 10 ppm for the precursor mass and 0.05 Da for fragment masses (Nesvizhskii *et al*., 2003).

Biocomputational analysis

To further elucidate the potential biological mechanisms and the complexity of AD pathology in terms of protein complexes and PPI, we used Ingenuity Pathway Analysis (IPA) algorithm (http://www.ingenuity.com) that deals with the specific biological task in a functional pathway due to change in the environment. Based on the identified interactions between proteins, IPA defines protein networks of the detected proteins. In addition, it also characterizes the canonical pathways altered by the differentially expressed proteins, thereby rendering additional information of the interacting proteins working in complexes.

Phosphorylation/ O-GlcNAc Modification Prediction Methods

In order to gain a deep insight of the processes related to AD progression, elucidation of PTM is necessary. The prediction method NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) for determining potential phosphorylation sites on differentially expressed proteins was used. This insilico method predicts serine, threonine and tyrosine phosphorylation sites on eukaryotic proteins derived from collective neural networks (Blom *et al*., 1999). The potential *O*-GlcNAc sites were predicted through YinOYang 1.2 (http//www.cbs.dtu.dk/services/YinOYang/). It is a prediction method based on neural network, comprised of numerous parallel working simulated neurons, to solve a complex problem. Both phosphorylation and *O*-GlcNAc (Yin O Yang sites) can be determined from the same software (Kaleem *et al*., 2011).

RESULTS

Identification of cortical protein complexes in control and AD brain

The protein complexes were isolated from human AD brain and age matched controls. After solubilization in dodecyl-maltoside they were separated on blue-native gel (Fig. **1**). In total thirteen protein complexes were detected on BN-PAGE with molecular masses ranging from 46 kDa to 715kDa obtained from Quantity One Software (Bio

Rad) (Table 1). Complex VII, comprised of molecular mass 305 kDa, containing highest number of interacting proteins along with significant differential expression was selected for further analysis.

Table 1. Apparent molecular weight of protein complexes obtained from the solubilization of human brain frontal cortical tissue homogenate (control and AD) analyzed by Quantity One software.

Complexes	MW kDa
I	715
II	699
III	602
IV	545
V	502
VI	453
VII	305
VIII	263
IX	149
X	130
XI	71
XII	53
XIII	46

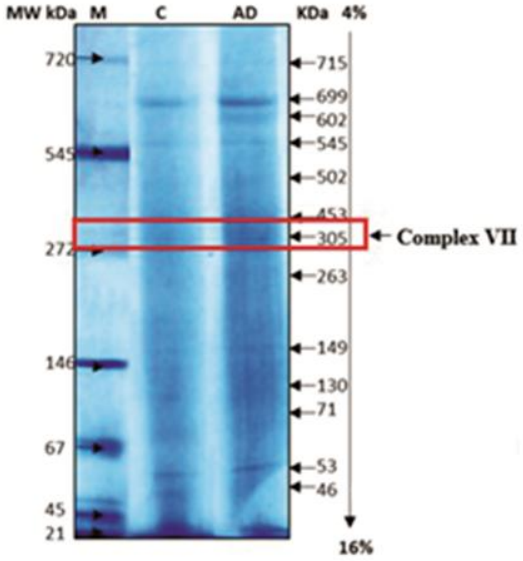

Fig. 1. Pattern of protein complexes (120µg) obtained from the separation of control and AD sample (lane 2 & 3) resolved on 1D BN-PAGE 4-16% gel. The protein bands are analyzed by Quantity One Software (Bio Rad).
M: Molecular weight marker
C: Control subjects
AD: Alzheimer's disease subjects.

Differential expression of protein components pertaining to complex VII

BN-PAGE was combined with denaturing SDS-PAGE to resolve the identities of components of the complex. The protein spots obtained were analyzed by nano LC-MS/MS and revealed seven proteins in this complex including GAPDH, ACTB, MAP1B, GFAP, PLP, COXII and ACPH. The molecular function, subcellular localization and predicted post translational modifications (PTMs) of the identified proteins associated in the form of complex were obtained through Gene Ontology (GO) and PANTHER databases (Fig. **2**) (Table 2). ACTB, GAPDH, ACPH, PLP1 represent increased expression and MAP1B illustrate decreased expression whereas GFAP and PLP1

remains unchanged in AD brain cortex in comparison with control. Major part of the interacting proteins identified, resides in the cytoplasm contributing in cytoskeletal assembly and metabolic homeostasis.

The fold change was obtained from Melanie 7.0 (GeneBio) image analysis software generated statistical analysis and significance ($p<0.05$) was calculated from Student's *t*-test.

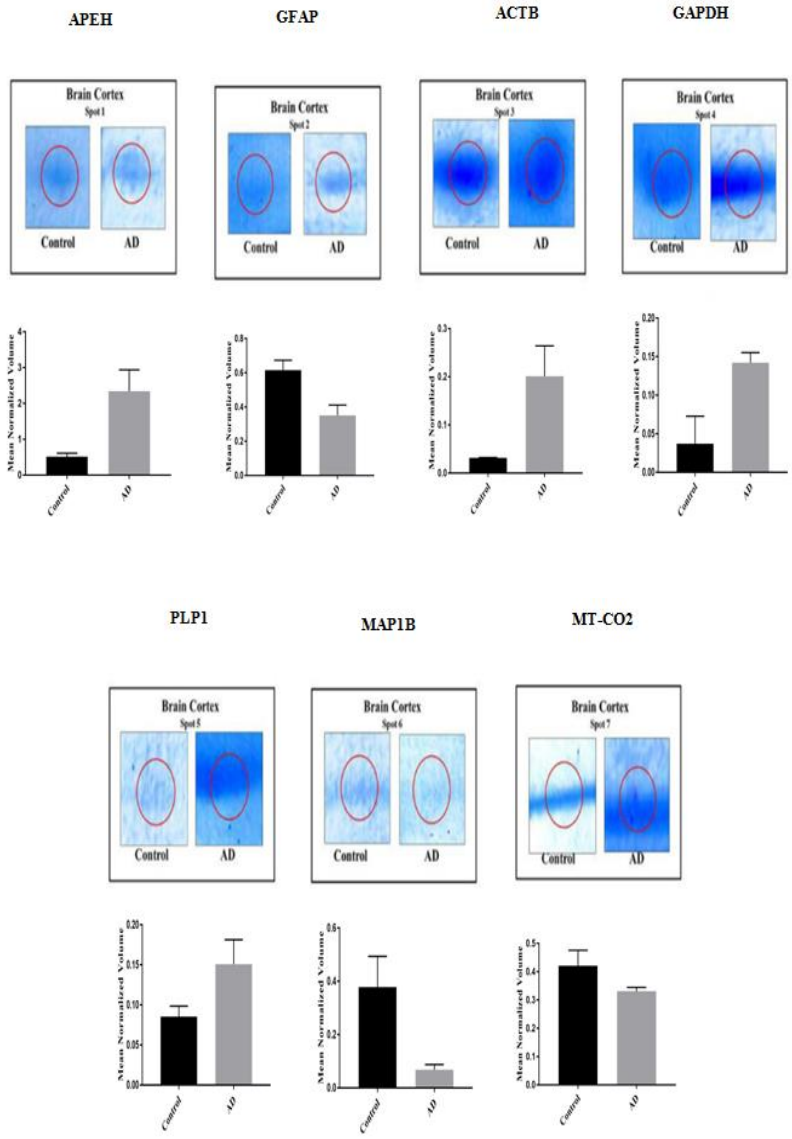

Fig. 2. Representative BN/SDS-PAGE spots and expression graphs acquired from separation of complex VII from control and AD brain frontal cortex. The interacting proteins of complex VII were separated on 12.5-7.5% SDS-PAGE in second dimension and Coomassie stained. The spot detection was performed with Melanie software version 7.0 (GeneBio) for 2D gel analysis. The expression graphs were generated using Graph Pad Prism 7 representing mean normalized volume in control and AD.

Canonical pathways and protein functional network analysis:

We next used the Ingenuity® Pathway Analysis (IPA®) (QIAGEN) tool to search for top biological functions, canonical pathways and protein networks that are affected in AD utilizing dataset of all dysregulated proteins based on propriety algorithm. The main representative molecular and cellular functions associated with our protein components include cell morphology (p-value 3.8E-02 – 1.14E-05), cellular movement (p-value 1.14E-02 – 1.89E-05), cellular development, growth and proliferation (p-value 3.90E-02 – 4.40E-05) and cell death and survival (4.2214E-02 – 1.12 E-04). The impaired molecular and cellular functions lead to hampered canonical pathways including NADH repair (p-value 9.99 E-4) glycolysis and gluconeogenesis (p-value 8.29E-3) and maturity onset

diabetes of young signaling (6.97 E-03,) disturbing the overall metabolism. One of the high-ranking pathways illustrates multidirectional interaction network of 35 proteins including 7 focus molecules. This network implicates several protein nodes directly or indirectly correlated with our identified proteins (Fig. **3**) (Table 3). The interacting proteins of the network are regulated by ACTB, GAPDH and TP53 creating interactive hubs and are associated with neurological diseases, skeletal and muscular disorders and metabolic diseases.

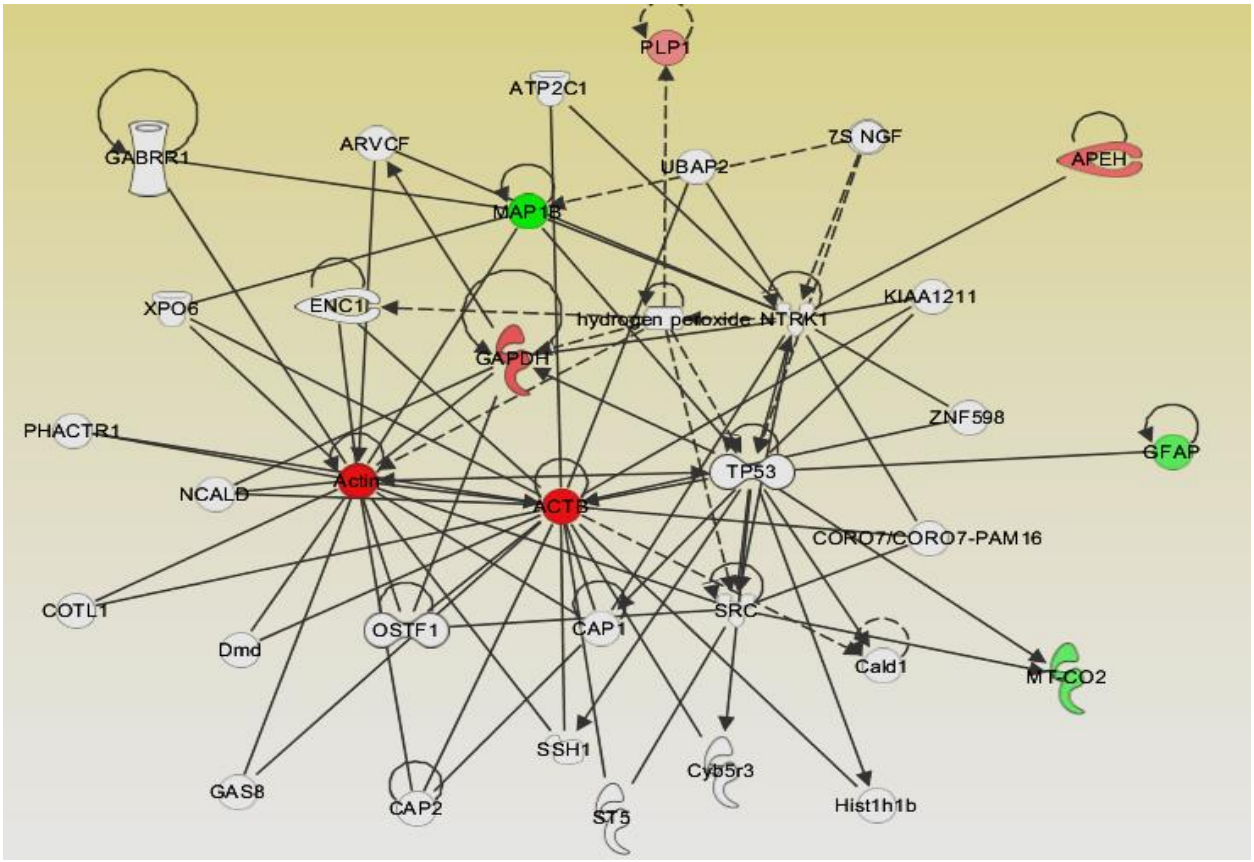

Fig. 3. Graphic representation of the top Ingenuity Pathway Analysis (IPA) generated network in AD. The IPA construct networks based on the differentially regulated proteins and their potential link with other known proteins in human AD prefrontal cortex. Known direct and indirect interactions between proteins of network, as well as the direction of the interaction, are indicated by arrows(activation) or blocked lines(inhibition). Central to the network are ACTB, GAPDH and TP53 altered in AD affecting most of the focus proteins resolved from protein complex.

Insilico analysis of phosphorylation sites in differentially expressed proteins

NetPhosK 3.1 server was used for the determination of potential kinase specific phosphorylation sites (http://www.cbs.dtu.dk/ services/NetPhosK/)(Blom *et al*., 2004). In order to eliminate false positive results, prediction with score below threshold 0.5 were neglected. The predicted phosphorylation sites of differentially expressed proteins are illustrated in Fig **4** (Table 4). Multiple phosphorylation sites on threonine serine and tyrosine aminoacids are obtained which needs further validation by experimental approaches.

Yin Yang sites of differentially expressed proteins

The possible Yin Yang sites, predicted by YinOYang 1.2 is depicted in Fig. **5** and given in Table 5. Sometimes Ser/Thr aminoacids illustrates a very high a potential for phosphorylation or *O*-GlcNAc modification. When a residue illustrates a very high potential, in proximity to the threshold value, for both phosphorylation and *O*-GlcNAc modification, it is taken as a false negative Yin Yang site as *O*-GlcNAc transferase (OGT) and kinase both may modify such a residue with equal chance. In ACPH Ser 245, 303, 304 and Thr 37, 276, 341, 498 in ACTB Ser 232 and 233 in GAPDH Ser 25, 284 and 333, in PLP Ser 194 are possible Yin Yang site with high threshold with the potential to undergo either phosphorylation and glycosylation. MAP1B due to its large peptide sequence has numerous Yin Yang sites.

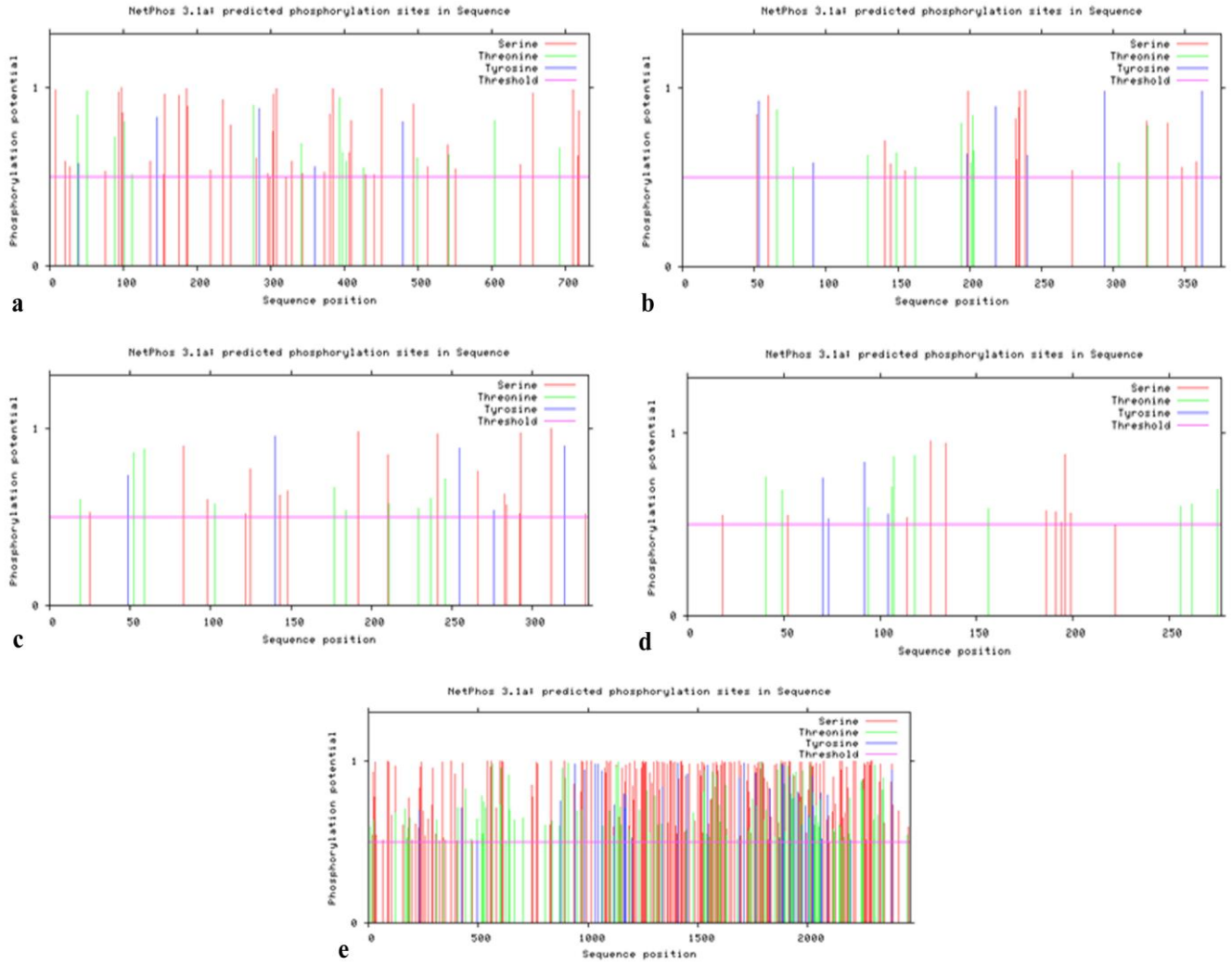

Fig. 4. Graphic presentation of potential phosphorylation sites on serine, threonine and tyrosine residues in a. APEH, b. ACTB, c. GAPDH, d. PLP1 and e. MAP1B sequences in control and AD brain frontal cortex obtained from NetPhos 3.1 server. Red, Green and Blue spectral lines represent serine, threonine and tyrosine, respectively.

DISCUSSION

Most of the proteomic strategies on neuronal system have so far either considered differential expression of proteins or their subcellular distribution in response to development or disease. Such studies lack information about the interaction configuration and PTM of these proteins, a very important aspect in the functioning of a protein. Capturing interacting protein partners of a complex in AD is made possible through BN-PAGE. In this study we employed MS based proteomic approach coupled with BN-PAGE for the identification of protein complexes. This provides for the first-time protein-protein interaction in cortical region of AD brain and implication of their interaction in disease pathology. One of the complex obtained on native PAGE with profound differential expression among control and AD brain was selected for further investigation and found to be comprised of seven interacting proteins validated by insilico analysis. The members of the complex identified in this study as diverse interacting partners in the human brain cortex are either cytoskeletal associates or metabolic enzymes. Moreover, the elicited pathways encompassing such proteins from different subcellular compartments provide a better understanding of AD based neuropathology.

Differentially expressed GAPDH interacts with almost all the components of the identified complex. We provide the evidence that GAPDH participates in regulation of crosstalk between microtubules and actin microfilaments through MAP1B. Interaction of GAPDH with the β-amyloid precursor protein (β-APP) (Cumming

and Schubert, 2005) , the β-amyloid protein (β-AP) (El Kadmiri *et al*., 2014) and tau (Wang *et al*., 2005) forming insoluble aggregates leads to cytotoxicity and indirectly triggers apoptosis (Wang *et al*., 2005, Cumming and Schubert, 2005). The increase in expression of GAPDH in AD brain observed in this study can be associated with an increased aggregation and accumulation of tau and Aβ in intracellular and extracellular regions. Obtaining GAPDH and oxidative phosphorylation enzyme COXII in a single complex as an interacting partner can be linked to initiation of apoptosis in AD. Considering the role of GAPDH accumulation in mitochondria as a proapoptotic protein (Tarze *et al*., 2007) and defects in OXPHOS enzymes during AD (Manczak *et al*., 2004). The interaction observed between GAPDH and GFAP is also a novel finding predicted for the first time in AD obtained through BN-PAGE. GFAP being a principal intermediate filament (IF) protein provides structural stability and expansion of astrocytic extensions thereby modulating its motility and shape (Hol and Pekny, 2015) which are degenerated during AD pathogenesis (Rohn *et al*., 2013). Myelin proteolipid protein (PLP) constituting ~50% of the total myelin protein content (Harlow *et al*., 2014) is associated with AβPP, Aβ1-42, and amyloid plaques in the AD brain cortex (Zhan *et al*., 2015). The interaction of PLP with GAPDH and GFAP observed predicts the involvement of other protein partners in AD hampering the normal axonal transport leading to cell death. Actin has been upregulated in AD cortex. Its polymerization is facilitated by GAPDH and has structural role in cellular assembly (Tristan *et al*., 2011). Beta actin, which interacts with multiple proteins for regulating their function, on oxidation may lead to mitochondrial instability culminating in AD pathogenesis (Sultana and Butterfield, 2009).

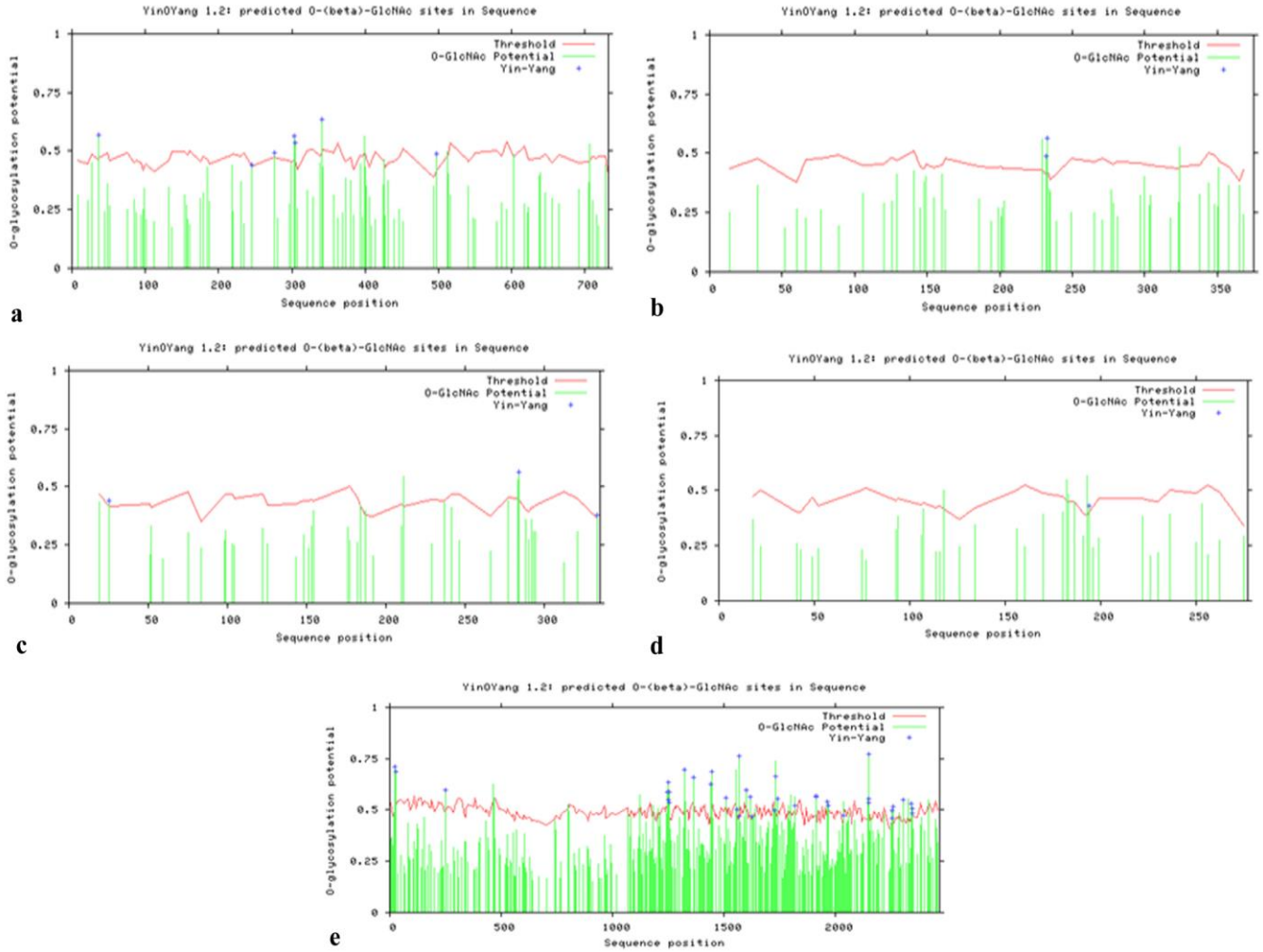

Fig. 5. Graphic presentation of potential for *O*-GlcNAc modification for Ser/Thr residues in a. APEH, b. ACTB, c. GAPDH, d. PLP1 and e. MAP1B sequences obtained from YinOYang 1.2 server in control and AD brain frontal cortex. Green vertical lines show the potential of Ser/Thr residue for *O*-GlcNAc modification and the red horizontal wavy line shows the threshold for modification potential.

Table 2. Proteins identified from complex VII by nano LC-MS/MS resolved on-SDS PAGE. Accession no. is obtained from SWISS/ Prot. Functional category and subcellular localization were determined by UniProt database (www.uniprot.org) and PANTHER databases (http://www.pantherdb.org/). The predicted PTMs were accessed from NetPhosK 3.1 (http://www.cbs.dtu.dk/services/NetPhosK). Group Based Prediction System for SNO 1.0 (GPS-SNO 1.0) (www.sno.biocuckoo.org) and UniProt (www.uniprot.org).

Accession No	Protein	Abb	M_r [kDa]	Score	Peptide Matched	Sequences	Functional Category	PTM	Subcellular location	Fold Change	p value
Complex VII											
P13798	Acyl amino acid releasing enzyme	APEH	81.1	80	2(1)	2(1)	Protein metabolism	Acetylation. phosphorylation	Cytoplasm	2.5	0.0099
P14136	Glial fibrillary acidic protein	GFAP	49.8	229	8	13(8)	Cytoskeleton	Citrullination. Phosphorylation	Cytoplasm. Intermediate filament	NS	0.014
P60709	Actin cytoplasmic	ACTB	41.7	187	10(6)	9(6)	Cytoskeleton	Acetylation. Methylation. Oxidation. Ubl conjugation	Cytoplasm, cytoskeleton	5.1	0.0044
P04406	Glyceraldehyde-3-PO_4DH	GAPDH	36.03	201	12(4)	7(2)	Metabolism	Acetylation, ADP-ribosylation, Methylation. Phosphoprotein, S-nitrosylatio, Ubl conjugation	Cytoplasm, Cytoskeleton. Membrane, Nucleus	2.9	0.007
P60201	Myelin proteolipid protein	PLP1	30.07 1	199	11(4)	7(3)	Myelin sheet structure.	Lipidation. Disulphide bond formation.	Cell membrane; Multi-pass membrane protein. Myelin membrane	2.11	0.0082
P46821	Microtubule associated protein 1B	MAP1B	27.04	56	3(2)	2(2)	Hydrolase. structural molecule. protein binding	Acetylation. Phosphorylation. S-nitrosylation	Cytoplasm, cytoskeleton. cell junction. synapse. dendritic spine	-3.4	0.0027
P00403	Cytochrome c oxidase subunit 2	MT-CO2	25.5	72	4(2)	2(2)	Respiratory chain enzyme.	Phosphorylation	Mitochondrion inner membrane. mitochondrion	NS	0.1399

Table 3. Protein network in Alzheimer's Disease. The 7 focus molecules of the network are derived from the list of original proteomic findings. IPA generates the network using a proprietary algorithm, and adds proteins not contained in the original dataset. The p-score is calculated by IPA and indicates the probability of finding 7 or more focus proteins a in a network of 35 molecules randomly selected from IPA's Global Molecular Network. The p-score is defined as p-score = -log10 (p-value); the p-value is calculated by Fisher's exact test.

Molecules in network	p-Score	Focus molecules	Top diseases and function
7S NGF, **ACTB**, Actin, **APEH**, ARVCF, ATP2C1, Cald1, CAP1, CAP2, CORO7/CORO7-PAM16, COTL1, Cyb5r3, Dmd, ENC1, GABRR1, **GAPDH**, GAS8, **GFAP**, Hist1h1b, hydrogen peroxide, KIAA1211, **MAP1B**, **MT-CO2**, NCALD, NTRK1, OSTF1, PHACTR1, **PLP1**, SRC, SSH1, ST5, TP53, UBAP2, XPO6, ZNF598	21	7	Cell Death and Survival, Cellular Assembly and organization, Cellular function and maintenance

Table 4. Prediction results of *O*-Phosphorylation sites of differentially expressed proteins in human AD brain cortex using NetPhos K 3.1 (http://www.cbs.dtu.dk/services/NetPhos/). The sites with p-value < 0.5 are not included.

Protein	Predicted phosphorylation sites		
	Serine	Threonine	Tyrosine
APEH	8, 21, 27, 75, 94, 97, 99, 136, 154, 156, 175, 185, 187, 218, 235, 245, 280, 296, 299, 303, 304, 308, 321, 329, 342, 373, 380, 384, 406, 409, 428, 440, 451, 493, 513, 540, 550, 639, 655, 710, 716, 718.	37, 51, 88, 101, 112, 276, 341, 393, 397, 399, 402, 414, 426, 432, 498, 541, 603, 692.	39, 145, 284, 360, 479.
ACTB	52, 60, 141, 145, 155, 199, 232, 233, 234, 235, 239, 271, 323, 338, 348, 358.	66, 77, 129, 149, 162, 194, 201, 202, 203, 304, 324.	53, 91, 198, 218, 240, 294, 362.
GAPDH	25, 83, 98, 122, 125, 143, 148, 192, 210, 241, 266, 283, 284, 292, 293, 312, 333	19, 52, 59, 103, 177, 184, 211, 229, 237, 246, 277, 290, 294	49, 140, 255, 276, 320
MAP1B	16, 23, 25, 29, 35, 65, 87, 91, 104, 121, 156, 185, 199, 216, 227, 234, 236, 241, 251, 258, 272, 288, 292, 306, 322, 336, 339, 343, 351, 377, 392, 401, 430, 431, 471, 541, 544, 554, 561, 582, 601, 609, 614, 742, 743, 765, 770, 828, 831, 832, 891, 936, 937, 965, 967, 970, 977, 992, 995, 1016, 1076, 1083, 1084, 1094, 1096, 1101, 1117, 1122, 1127, 1128, 1137, 1144, 1154, 1156, 1168, 1171, 11887, 1190, 1205, 1208, 1211, 1212, 1219, 1221, 1234, 1244,1247, 1248, 1252,1254, 1256, 1258, 1260, 1262, 1265, 1280, 1289, 1298, 1312, 1322, 1324, 1326,	9, 24, 122, 167, 177, 178, 187, 230, 310, 340, 405, 444, 467, 494, 517, 522, 527, 533, 565, 598, 603, 626, 637, 642, 667, 704, 804, 837, 870, 885, 897, 898, 899, 908, 939, 948, 972, 1067, 1099, 1116, 1131, 1138, 1147, 1152, 1192, 1235, 1270, 1282, 1302, 1317, 1334, 1449, 1478, 1525, 1532, 1542, 1554, 1566, 1567, 1584, 1597, 1599, 1600, 1628, 1633, 1680, 1712, 1732, 1737, 1749, 1766, 1788, 1804, 1813, 1853, 1864, 1878, 1879, 1895, 1898, 1912, 1913, 1925, 1929, 1930, 1946, 1947, 1949, 1963, 1964, 1977, 2010, 2011, 2015,	232, 423, 496, 876, 942, 985, 1034, 1044, 1062, 1119, 1165, 1170, 1174, 1200, 1337, 1410, 1454, 1543, 1689, 1711, 1762, 1830, 1872, 1887, 1889, 1892, 1921, 1923, 1938, 1940, 1955, 1974, 1991, 206, 2023, 2025, 2059, 2066, 2090, 2195, 2385

	1339, 1346, 1362, 1366, 1376, 1378, 1387, 1389, 1396, 1400, 1406, 1408, 1412, 1415, 1421, 1427, 1433, 1438, 1443, 1446, 1452, 1462, 1486, 1501, 1508, 1512, 1520, 1522, 1527, 1530, 1544, 1551, 1556, 1560, 1561, 1571, 1573, 1580, 1583, 1589, 1593, 1607, 1609, 1618, 1620, 1625, 1631, 1644, 1646, 1653, 1666, 1690, 2692, 1697, 1698, 1700, 1722, 1724, 1729, 1731 1732.3, 1734, 1739, 1743, 1760, 1764, 1767, 1772, 1779, 1782, 1785, 1793, 1797, 1803, 1805, 1816, 1818, 1819, 1826, 1835, 1852, 1860, 1869, 1881, 1891, 1899, 1915, 1917, 1919, 1922, 1939, 1956, 1960, 1965, 1970, 1973, 1980, 1994, 2005, 2007, 2020, 2024, 2028, 2038, 2055, 2058, 2062, 2072, 2074, 2086, 2098, 2100, 2112, 2119, 2126, 2149, 2151, 2153, 2156, 2160, 2171, 2180, 2184, 2209, 2211, 2218, 2254, 2255, 2256, 2261, 2265, 2271, 2281, 2285, 2289, 2295, 2329, 2331, 2347, 2348, 2382, 2383, 2388, 2414, 2460.	2027, 2029, 2031, 2034, 2039, 2049, 2051, 2056, 2061, 2116, 2124, 2145, 2148, 2158, 2166, 2186, 2192, 2194, 2243, 2245, 2250, 2252, 2303, 2304, 2305, 2320, 2341, 2344, 2453.
PLP1	18, 52, 114, 151, 156, 159, 161, 164, 187	41, 49, 94, 106, 107, 116, 221, 227, 240	70, 73, 92, 104

Table 5. Prediction results of *O*-Glycosylation sites of differentially expressed proteins in human AD brain cortex using YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/)

Protein	Predicted sites for O-β-GlcNAc		
	Serine	**Threonine**	
APEH	245, 303, 304, 512	36, 37, 276, 341, 399, 426, 498, 603, 706	
ACTB	14, 33, 52, 60, 41,145,155,199, 232*, 233*, 234, 235, 239, 265, 271, 281, 300, 323, 338, 344, 348, 350, 358, 365, 368.	66, 77, 89, 106, 120, 126, 129, 148, 149, 160, 162, 186, 194, 201, 202, 203, 229, 249, 277, 278, 297, 303, 304, 318, 324, 351	
GAPDH	25*, 283, 284*, 333*	184,187, 211	
MAP1B	16, 23*, 25*, 251*464, 470, 1247*, 1248*, 1252*, 1254*, 1256*, 1322, 1362*, 1443*, 1446*, 1508*, 1553, 1560*, 1618*, 1625*, 1631, 1640, 1729*, 1733*, 1739*, 1792, 1801, 1816*, 18171915*, 1970*, 2149*, 2151*, 2254*, 2255*, 2261*, 2327, 2347*.	24, 801, 1123, 1181, 1206, 1328, 1554, 1566*, 1567*, 1600*1732, 1913*, 1964*, 2034, 2037, 2145, 2148, 2158, 2250, 2304*, 2341*, 2342, 2344*, 2345, 2419,	
PLP1	186, 194*	118, 182, 183, 193	

* YinYang sites (Predicted residues having potential for both phosphorylation and *O*-GlcNAc)

The present study is in accordance with the previous findings of differential expression of these proteins in AD including ACTB, GAPDH, MAP1B (Yokota *et al*., 2006) , GFAP (Oddo *et al*., 2003) and ACPH (Yamin *et al*., 2009). However, the functional diversity of a protein cannot be exclusively explained by the frequency of expression rather the interacting partners of the protein working in a complex association. Therefore, using IPA analysis, construction of the entire set of complex interaction among proteins involved on AD was performed. A striking observation is TP53 making a hub with GAPDH and ACTB in one of the main networks, interacting with all the proteins of complex VII. It is a tumor suppressor protein causing apoptosis by arresting the cell cycle (Hooper *et al*., 2007), thus predicting the role of differentially expressed proteins in apoptotic pathway terminating in AD progression. NADH repair is the top canonical pathway with highest p-value highlighting the importance of GAPDH in Alzheimer's disease.

The interaction between the seven proteins of the complex with each other suggests that instead of predicting interaction between each pair of protein independently we try to work on them collectively to form functional pathways that carryout most cellular processes. Such higher level of interactions are highly dependent on PTMs of proteins. In particular PTM disrupt the interaction often changing the behavior of the corresponding pathways leading to a change in phenotype. The alternate phosphorylation and glycosylation are PTMs that regulate the function and properties of proteins. The interplay between these two modifications on the neighboring or same residues intervene in various functional activities (Kaleem *et al*., 2009). Glycosylation, important in cellular development and homeostasis (Bacigalupa *et al*., 2018). This modification has been reported in some proteins of AD including APP, tau, presenilin, BACE1, acetyl choline esterase and nicastrin (Schedin-Weiss *et al*., 2014) but still many proteins needs further investigation for their glycosylation sites. The possible sites on our differentially expressed proteins predicts their involvement in AD pathogenesis on alteration. Protein phosphorylation is a regulatory mechanism for various cellular processes and its aberrations has been linked to AD (Henriques *et al*., 2016). One of our earlier studies on AD have identified phosphorylation of GAPDH, ACPH and GFAP along with nitrosylation of actin regulating their biological activity (Zahid *et al*., 2014, Zahid *et al*., 2012). Phosphorylation of GAPDH modulates its actin and MAP1B binding, in turn affecting the actin polymerization and microtubule bundling (Cueille *et al*., 2007). Although MAP1B and tau exhibits hyperphosphorylatin in AD, it has been observed that normal MAP1B can also be sequestered by pathologic tau in AD brain resulting in inhibition and disruption of microtubules (Gong and Iqbal, 2008). Because of the observed association of GAPDH with beta actin, MAP1B, GFAP and PLP in this study, it is postulated that GAPDH may modulate the cytoskeleton structure and dysfunctioning of the protein complex and interacting partners leading to progression of cell death in AD.

CONCLUSION

Protein complexes are identified in control and AD brain and their clinico-pathological association has been revealed leading to a better understanding of how a given protein may function with the contact of a network of other proteins or specific pathway. Here we have concentrated on expression pattern, interaction partners and potential phosphorylation and *O*-GlcNAc of these proteins, but clearly more studies are needed to clarify their physiological importance during interaction with each other.

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REFERENCES

Atanassov, I. and H. Urlaub (2013). Increased proteome coverage by combining PAGE and peptide isoelectric focusing: Comparative study of gel-based separation approaches. *Proteomics*, 13**:** 2947-2955.

Bacigalupa, Z. A., C.H. Bhadiadra and M.J. Reginato (2018). O-GlcNAcylation: key regulator of glycolytic pathways. *Journal of Bioenergetics and Biomembranes***,** p.1-10. doi.org/10.1007/s10863-018-9742-3

Blom, N., S. Gammeltoft and S. Brunak (1999). Sequence and structure-based prediction of eukaryotic protein phosphorylation sites1. *Journal of molecular biology*, 294**:** 1351-1362.

Blom, N., T. Sicheritz-Pontén, R. Gupta, S. Gammeltoft and S. Brunak (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics*, 4**:** 1633-1649.

Chang, J. R., M. Ghafouri, R. Mukerjee, A. Bagashev, T. Chabrashvili and B. E. Sawaya (2012). Role of p53 in neurodegenerative diseases. *Neurodegenerative Diseases*, 9**:** 68-80.

Cueille, N., C. T. Blanc, S. Popa-Nita, S. Kasas, S. Catsicas, G. Dietler and B. Riederer (2007). Characterization of MAP1B heavy chain interaction with actin. *Brain research bulletin*, 71**:** 610-618.

Cumming, R. C. and D. Schubert (2005). Amyloid-β induces disulfide bonding and aggregation of GAPDH in Alzheimer's disease. *The FASEB journal*, 19**:** 2060-2062.

El Kadmiri, N., I. Slassi, B. El Moutawakil, S. Nadifi, A. Tadevosyan, A. Hachem and A. Soukri.(2014). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Alzheimer's disease. *Pathologie Biologie*, 62**:** 333-336.

Frenkel-Pinter, M., M.D. Shmueli, C. Raz, M. Yanku, S. Zilberzwige, E. Gazit and D. Segal (2017). Interplay between protein glycosylation pathways in Alzheimer's disease. *Science advances*, 3**:** e1601576.

Gong, C.-X. and K. Iqbal (2008). Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease. *Current Medicinal Chemistry*, 15**:** 2321.

Harlow, D. E., K.E. Saul, C. M. Culp, E. M. Vesely and W. B. Macklin (2014). Expression of proteolipid protein gene in spinal cord stem cells and early oligodendrocyte progenitor cells is dispensable for normal cell migration and myelination. *The Journal of Neuroscience*, 34**:** 1333-1343.

Hayes, S., B. Malacrida, M. Kiely and P. A. Kiely (2016). Studying protein–protein interactions: progress, pitfalls and solutions. *Biochemical Society Transactions*, 44**:** 994-1004.

Henriques, A. G., T. Müller, J. M. Oliveira, M.E. Cova, C. B. D. C. E-Silva and O. A. D. C. E-Silva (2016). Altered protein phosphorylation as a resource for potential AD biomarkers. *Scientific reports*, 6**:** 30319.

Hol, E. M. and M. Pekny (2015). Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system. *Current opinion in Cell Biology*, 32**:** 121-130.

Hooper, C., E. Meimaridou, M. Tavassoli, G. Melino, S. Lovestone and R. Killick (2007). p53 is upregulated in Alzheimer's disease and induces tau phosphorylation in HEK293a cells. *Neuroscience letters*, 418**:** 34-37.

Kaleem, A., D.C. Hoessli, I. U. Haq, E. Walker-Nasir, A. Butt, Z. Iqbal, Z. Zamani and A. R. Shakoori (2011). CREB in long-term potentiation in hippocampus: Role of post-translational modifications-studies In silico. *Journal of cellular biochemistry*, 112**:** 138-146.

Kaleem, A., I. Ahmad, E. Walker-Nasir, D. C. Hoessli and A.R. Shakoori (2009). Effect on the Ras/Raf signaling pathway of post-translational modifications of neurofibromin: In silico study of protein modification responsible for regulatory pathways. *Journal of cellular biochemistry*, 108**:** 816-824.

Klapa, M. I., K. Tsafou, E. Theodoridis, A. Tsakalidis and N.K. Moschonas (2013). Reconstruction of the experimentally supported human protein interactome: what can we learn? *BMC systems biology*, 7**:** 96.

Lee, S.-A, T. T.-H. Tsao, K.-C. Yang, H. Lin, Y.-L. Kuo, C.-H. Hsu, W.-K. Lee, K.-C. Huang and C.-Y. Kao (2011). Construction and analysis of the protein-protein interaction networks for schizophrenia, bipolar disorder, and major depression. BMC bioinformatics, 2011. BioMed Central, S20.

Malhotra, A., E. Younesi, S. Sahadevan, J. Zimmermann and M. Hofmann-Apitius (2015). Exploring novel mechanistic insights in Alzheimer's disease by assessing reliability of protein interactions. *Scientific reports*, 5**:** 13634.

Manczak, M., B.S. Park, Y. Jung and P. H. Reddy (2004). Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease. *Neuromolecular Medicine*, 5**:** 147-162.

Nesvizhskii, A. I., A. Keller, E. Kolker and R. Aebersold (2003). A statistical model for identifying proteins by tandem mass spectrometry. *Analytical chemistry*, 75**:** 4646-4658.

Oddo, S., A. Caccamo, M. Kitazawa, B.P. Tseng and F.M. Laferla (2003). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiology of aging*, 24**:** 1063-1070.

Rohn, T. T., L. W. Catlin and W. W. Poon (2013). Caspase-cleaved glial fibrillary acidic protein within cerebellar white matter of the Alzheimer's disease brain. *International Journal of Clinical and Experimental Pathology*, 6**:** 41.

Schägger, H. (2006). Tricine–SDS-PAGE. *Nature Protocols*, 1**:** 16-22.

Schedin-Weiss, S., B. Winblad and L. O. Tjernberg (2014). The role of protein glycosylation in Alzheimer disease. *The FEBS journal*, 281**:** 46-62.

Sultana, R. and D. A. Butterfield (2009). Oxidatively modified, mitochondria-relevant brain proteins in subjects with Alzheimer disease and mild cognitive impairment. *Journal of bioenergetics and biomembranes*, 41**:** 441-446.

Talwar, P., Y. Silla, S. Grover, M. Gupta, R. Agarwal, S. Kushwaha and R. Kukreti (2014). Genomic convergence and network analysis approach to identify candidate genes in Alzheimer's disease. *BMC genomics,* 15**:** 199.

Tarze, A., A. Deniaud, M. Le Bras, E. Maillier, D. Molle, N. Larochette, N. Zamzami, G. Jan, G. Kroemer and C. Brenner (2007). GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. *Oncogene,* 26**:** 2606-2620.

Tristan, C., N. Shahani, T.W. Sedlak and A. Sawa (2011). The diverse functions of GAPDH: views from different subcellular compartments. *Cellular signalling,* 23**:** 317-323.

Wang, Q., R. L. Woltjer, P. Cimino, C. Pan, K.S. Montine, J. Zhang and T. J. Montine (2005). Proteomic analysis of neurofibrillary tangles in Alzheimer disease identifies GAPDH as a detergent-insoluble paired helical filament tau binding protein. *The FASEB Journal,* 19**:** 869-871.

Wittig, I., H.-P. Braun and H. Schägger (2006). Blue native PAGE. *Nature Protocols,* 1**:** 418-428.

Yamin, R., C. Zhao, P.B. O'connor, A. C. Mckee and C. R. Abraham (2009). Acyl peptide hydrolase degrades monomeric and oligomeric amyloid-beta peptide. *Molecular neurodegeneration,* 4**:** 33.

Yokota, T., M. Mishra, H. Akatsu, Y. Tani, T. Miyauchi, T. Yamamoto, K. Kosaka, Y. Nagai, T. Sawada and K. Heese (2006). Brain site-specific gene expression analysis in Alzheimer's disease patients. *European journal of clinical investigation,* 36**:** 820-830.

Zahid, S., R. Khan, M. Oellerich, N. Ahmed and A. R. Asif (2014). Differential S-nitrosylation of proteins in Alzheimer's disease. *Neuroscience,* 256**:** 126-136.

Zahid, S., M. Oellerich, A.R. Asif and N. Ahmed (2012). Phosphoproteome profiling of substantia nigra and cortex regions of Alzheimer's disease patients. *Journal of Neurochemistry,* 121**:** 954-963.

Zhan, X., G. C. Jickling, B. P. Ander, B. Stamova, D. Liu, P.F. Kao, M. A. Zelin, L.-W. Jin, C. Decarli and F.R. Sharp (2015). Myelin Basic Protein Associates with AβPP, Aβ 1-42, and Amyloid Plaques in Cortex of Alzheimer's Disease Brain. *Journal of Alzheimer's disease,* 44**:** 1213-1229.

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