Study of biomarkers involved in amyloidgenic pathway for over-production of amyloid beta leading to alzheimer's disease.

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Abstract

APP is an Amyloid Precursor Protein which is present endogenously in many parts of the body, is cleaved by enzyme β-secretase (a transmembrane aspartic protease enzyme- BACE). When APP is cleaved by this enzyme, the protein is divided into two parts i.e. Soluble sAPPβ and membrane bound CTFβ. There is an enzyme γ-secretase which cleaves this CTFβ structure, dividing into Amyloid Beta peptides and membrane bound fragment AICD. Since, Amyloid beta (Aβ) is required for neural functioning but Cluster formation of these Amyloid beta peptides leads to form plaques that interrupts the Brain function, finally causing neural cell death. These plaques disturb the brain cells by clogging the cell junctions and hence activating immune cells for inflammation which become lethal to cells. Hence, two key biomarkers are studied with the help of Western Blotting to check their activity and over production in normal cells so as to study the change after being diseased.

Keywords: Amyloid precursor, Neuro-degenerative disease, Amyloidogenic.

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Introduction

According to 2019 Alzheimer's Association statistics, 4 Million patients Alzheimer's disease is a neuro-degenerative disease and is considered as a Global public Health Priority by WHO. The very first case was seen in 1907, around a century ago but there is no treatment by far now. Dementia is a cognitive impairment that leads to memory loss, personality loss, etc. This is because nerve cells that work in cognitive responses have been damaged or destroyed. Worldwide, 44 million individuals have dementia and it is estimated that by the end of 2050, the count exceeds to triple of the today's condition. Alzheimer's disease accounts for 50-75% of the dementia cases worldwide. Studies concluded that old age group with dementia have brain abnormalities have more than one cause of dementia, hence known as mixed dementia. It is believed that early detection of the disease can be very helpful in curing. Last 10 years have shown a tremendous success in the field. Inherited form of Alzheimer's disease can be characterized by early occurrence around or before 50 years and is due to the mutations in APP gene or gene codes for protein PS1 and PS2 which are the components of γ-secretase complex.

Alzheimer's disease is a neurodegenerative disease which is caused due to plaque formation of Amyloid Precursor protein. Plaques are formed when 40-42 APP polymerise. This disease can be caused by Arctic Mutation in the APP. Amyloid beta is thought to be the autosomal dominant form in Alzheimer's. The disease is caused in Amyloidogenic Pathway in the body. The major components for the cause of Alzheimer's are Amyloid beta which is produced from APP by the action of enzymes like β-secretase and γ-secretase. B-secretase cleaves the APP to the N-terminal and this leads to the formation of Amyloid beta peptides. The accumulation of these peptides leads to the formation of Plaque. BACE is being knocked out in mice and then studied for pathology, their activity and Plaque formation. It was examined that when BACE is knocked out into the mice, the mice is healthy but β-secretase lacks in their brain. B-secretase activity is not detectable in 1 Cortical Culture and in the brains of knocked out mice. It was indicated that pharmacological inhibition of BACE will stop the production of Amyloid beta peptide. Only small amount of these inhibitors can inhibit the formation of the plaques and prevent the growth of pre-existing Plaques too. Hence, it was concluded that BACE is the key enzyme that leads to the formation of these peptide and studies suggests that it is the best therapeutic target for Alzheimer's disease [1].

Materials and Methods

BACE is novel transmembrane aspartic protease, has a pro-peptide domain that is used to form mature enzymes. Mutations in these genes, APP, PS1 and PS2 mainly lead to Alzheimer's. APP is large Type I Membrane protein. γ-secretase enzyme has been identified as PS1 and PS2, and β-secretase as BACE. BACE can be classified as BACE 1 which is commonly known as Asp2 and memapsin2, and BACE 2 is homologous to BACE 1. BACE 1 is key enzyme that initiates the Aβ formation. BACE 1 is the key acceptor for Drug target. β-secretase is present in almost all the cells and tissue of the body while there maximum activity is found to be activated in neural tissues and neuronal cell line in acidic ph. The active site of this enzyme is present within the lumen of acidic intracellular compartments (Figure 1).

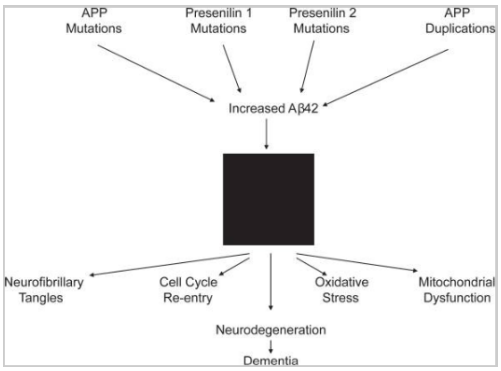

Figure 1. *Causes and result of alzheimer's disease.*

Characterisation of β-secretase Protein BACE suggests their function and the overall formation of plaques. Experiments were done for their characterisation. The process involves first Analysis of BACE membrane binding of 293 cells by isolation the membrane bound protein. Their presence was estimated by washing with 0.5M NaCl. It is followed with Analysis of Post Translational Modification of BACE. A Polyclonal Ab specific to BACE's pro-peptide region was made to perform Immuno-precipitation. Now, BACE-IgG is prepared and purified. It is then treated with Fluorescein 5-Maleimide followed by its proteolytic fragmentation. Finally, the peptides are separated by HPLC. For characterisation, Mass Spectrometry is done using MALDI Technique. Finally, Carbohydrate and Amino Acid Sequence Analysis is done.

BACE1 mediates the essential amyloidogenic cleavage of APP and produces APP C-terminal section (APP CTFb), which is the quick precursor for the intra-membranous γ-secretase cleavage. BACE-1 is the main protease with well-defined β-secretase action. BACE 1 is generated as prepoenzyme. After the action of BACE 1, γ-secretase starts functioning which is required for unusual intermembrane cleavage events. Presenilins i.e. PS1 and PS2 plays an important role in γ-secretase cleavage events. The over-expression of this particular protease which is termed as BACE (β-site APP Cleaving Enzyme) led to the increase in the amount of all β-secretase activities. It was then concluded that the Cellular localization and Expression pattern of BACE is consistent except with β-secretase [2]. The major component for the cause of Alzheimer's disease is Amyloid beta which is produced from APP by the action of enzymes like β-secretase and γ-secretase. β-secretase cleaves the APP to the N-terminal and this leads to the formation of Amyloid beta peptides. The accumulation of these peptides leads to the formation of Plaque. Characterization of β-secretase Protein BACE suggests their function and the overall formation of plaques. Experiments were done for their characterization. The process involves Analysis of BACE membrane binding of 293 cells by isolation the membrane bound protein. Their presence was estimated by washing with 0.5M NaCl. It is followed with Analysis of Post Translational Modification of BACE. A Polyclonal Ab specific to BACE's pro-peptide region was made to perform Immuno-precipitation. Now, BACE-IgG is prepared and purified. It is then treated with Fluorescein 5-Maleimide followed by its proteolytic fragmentation. Finally, the peptides are separated by HPLC. For characterization, Mass Spectrometry is done using MALDI Technique. Finally, Carbohydrate and Amino Acid Sequence Analysis is done. Survey was conducted to check the activity of different enzymes, Patients having sporadic Alzheimer's disease shown an alteration of APP form expressions in platelets when compared with non-AD patients. Decrease in α-secretase activity is also noticed. The decrease in end proteolytic components recommends that there must be an uplifted action of the active BACE structures. An increased BACE action was recently proposed by estimations performed in after death tissues.

Mutations can be the reason for Alzheimer's disease. Earlier, it was estimated that the mutation at codon 717 in exon 17 of β-APP gene. But, mutation at codon 670 and 671 in exon 16 also lead to this disease. There is two base pair transversion change in the sequence that changes Lys to Asn and Met to Leu Amino Acid. So, it was thought that there is some pathogenic mutation for the disease in APP gene at β-amyloid's N-terminal. Amyloid-β activation of the NLRP3 inflammasome in microglia is key for interleukin-1β development. In any case, it stays unknown whether NLRP3 activation adds to Alzheimer's infection in vivo. Here we exhibit emphatically improved active caspase-1 articulation in human brain with Alzheimer's disease, recommending a role for the inflammasome in this neurodegenerative infection. Mice carrying mutation related with familial Alzheimer's ailment were to a great extent protected from loss of spatial memory and other sequelae related with Alzheimer's infection, and exhibited diminished mind caspase-1 and interleukin-1β activation just as upgraded amyloid-β leeway. Moreover, NLRP3 inflammasome inadequacy skewed microglial cells to a M2 phenotype and brought about the diminished affidavit of amyloid-β in the APP/PS1 model of Alzheimer's clearance. These outcomes demonstrate an imperative role for the NLRP3/caspase-1 axis in the pathogenesis of Alzheimer's malady, and recommend that NLRP3 inflammasome inhibition represents to another helpful intercession for the disease. Evidence that exists shows that Neuroinflammation might cause Alzheimer's disease. Misfolded and clustered proteins bind to pattern recognition receptors on microglia and astroglia, and trigger an innate response reaction described by release of inflammatory mediators, which add to disease movement. GWS recommends that few genes that increase the risk for sporadic Alzheimer's infection encode factors that control glial freedom of misfolded proteins and the inflammatory response.

Alzheimer's disease, the most common reason of dementia, is still defined by the presence of amyloid and tau, yet researchers are working endlessly from the simple suspicion of linear causality as proposed in the first amyloid hypothesis. Age-related, protective, and disease promoting components most likely interact with the core systems of the AD. Amyloid β42, and tau proteins are set up core cerebrospinal biomarkers; novel biomarkers include amyloid β oligomers and synaptic markers.

Lately, it has been reported that olfactory dysfunction is also associated with AD but not much explored, about the

relationship between olfaction and neural specific substrates mainly in people above 50 years. Clinical olfactory testing must be done because it will provide a lot of knowledge about the progression rate of aMCI (amnestic mild cognitive impairment) to Alzheimer's disease. Metabolic Syndromes such as high Blood Pressure, high Sugar level, mainly Obesity and Insulin resistance are much risk factors for AD. Many developments have been coming across the different approaches such as NGS and microarray. One approach via Systems Biology is made to discover novel micro-RNA and gene based biomarkers for the diagnosis [3].

Cytokines majorly involved in the early progression of AD are interleukin-1α (IL-1α), IL-β, IL-6, and tumor necrosis factor-α (TNF-α) in which IL-1 is responsible in the development of Alzheimer's Disease. Increased levels of IL-1 has been found in AD patient's brain and cerebrospinal fluid. Astrocytes helps in forming the structure of brain, constituting almost 30%-55% of the brain. It plays an important role in maintaining normal function of the brain and in neural disorders. These cells is associated with progression of cells. Chemokines CCL2 and CCL3 promotes their migration towards amyloid plaques hence mediate neurotoxicity and become active in clearance of Aβ.

One Survey was conducted to check the activity of different enzymes, Patients having sporadic Alzheimer's disease shown an alteration of APP form expressions in platelets when compared with non-AD patients. Decrease in α-secretase activity is also noticed. The decrease in endoproteolytic components recommends that there must be an uplifted action of the active BACE structures. An increased BACE action was recently proposed by estimations performed in after death tissues. Mutations can be the reason for Alzheimer's disease. Earlier, it was estimated that the mutation at codon 717 in exon 17 of β-APP gene. But, mutation at codon 670 and 671 in exon 16 also lead to this disease. There is two base pairs transverion change in the sequence that changes Lys to Asn and Met to Leu Amino Acid. So, it was thought that there is some pathogenic mutation for the disease in APP gene at β-amyloid's N-terminal (Figure 2).

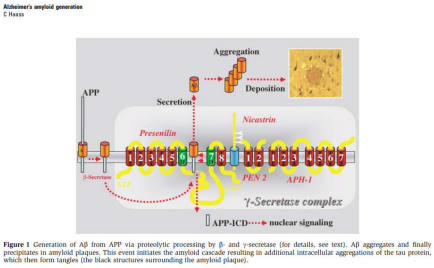

Figure 1 Generation of Aβ from APP via proteolytic processing by β- and γ-secretase (for details, see text). Aβ aggregates and finally precipitates in amyloid plaques. This event initiates the amyloid cascade resulting in additional intracellular aggregations of the tau protein, which then form tangles (the black structures surrounding the amyloid plaque).

Figure 2. *Formation of amyloid beta plaques by the proteolytic action of β- and γ-secretase.*

Western Blotting is used to separate and identify different proteins on the basis of their molecular weight by gel electrophoresis. The protein samples are then transferred onto the nitrocellulose or PVDF Membrane which imprints the bands of the respective protein. The proteins are then incubated with label antibodies which are specific to respective protein. The unbound Antibody is washed off leaving the space for secondary antibody to bind. The bounded antibody is then detected by developer on X-ray Film. Since, Antibodies only bind to proteins, so only specific band are visible on the film. The thickness and intensity of the band indicates the concentration of the protein, hence used to calculate the amount of protein.

It has been seen that different origins of stem cells can be useful in neurodegenerative disorders in animal models. Clinical studies going on suggests safety and effective results. Many studies have been hypothesized, some of them describes the release of neurotrophic factors by paracrine from stem cells while other shows immunodulatory effects. Manny studies reported the transplanted stem cells have the ability to replace diseased cells. While working in Animal models of AD, it is concluded that Expression of Synaptic proteins has been increased.

In the early onset of AD, it has been seen that there is hypo-metabolism which triggers hippocampus, cortex and neocortical regions in the brain. This leads to overexpression of amyloid beta proteins, leading to inflammation followed by oxidative stress and hence Neural Cell Death. HIF-1α is one of the major factor that is effective in inducing several proteins which is associated with nitric oxide synthase and erythropoietin function. Hence, maintaining HIF-1α level is important to cure nerve cells. Iron chelators, Cobalt or Nickel are found to be effective in maintaining its level. One of the recent research stated that mitochondrial dysfunction is associated with Alzheimer's disease. Damaged mitochondria accumulation is one of the symptom in AD. Since, Mitochondria is damaged, there is bioenergetics deficiency and oxidative stress in the nerve cells.

An endocrine hormone called, Melatonin, produced by pineal gland, is amphipathic molecules that has the ability to cross blood brain barrier. This hormone has an ability to enter subcellular organelles such as Mitochondria or ER. Melatonin can act as Antioxidant, Neuro-protectant by regulating the enzymes and Inhibition of Circadian Disruption by reducing amyloid beta accumulation and hyper-phosphorylation of tau molecules with the help of glycogen synthase kinase-3 (GSK-3) and CDK-5 signaling pathways. Around 65-75% AD cases happen because of genetic factors. Apolipoprotein E, mainly APOE-4 is considered as major factor in later onset of the disease. Several changes occur in white matter of the brain including loss of axons, interstitial fluid draining failure, etc [4].

Magnetoencephalography (MEG) is a technique with no involvement of instrument in the brain, to record neural activities by measuring magnetic fields which are oscillating. Due to limited clinical availability, the potential utilization of the technique is still unpredictable, though, it can be evolved in coming years in respect with Alzheimer's disease with great advancement. Diabetes, one of the most dangerous metabolic disease is found to be associated with AD in recent studies. The dysregulation of Insulin is a reason behind AD. Various patho-physiological process causing Alzheimer's disease are

Cerebral hypo-fusion, decreased vasoreactivity, loss of perivascular innervation, aging and vascular risk factors, amyloid beta accumulation, and impaired clearance of amyloid beta peptides from the brain. Recently, dysregulation of Calcium is involved in physio-pathology of AD. Calcium acts as important messengers in regulation of various signaling pathways, which involved in memory formation and many physiological functions. G-Protein Coupled Receptors release secondary messengers which regulates Calcium Homeostasis, hence can maintain their regulation the nerve cells. Aβ and tau proteins pathology is associated with circadian rhythms disturbances and prominently contributing to sleep dysfunction. With the progression of AD, they confirms their mutual dependent pathways. Further, these rhythms is linked with different pathways and systems of the body.

Currently used Medication method includes two types of Drugs that act upon memory symptoms and other body changes. One of them is Cholinesterase Inhibitors that helps in boosting cell-cell interactions by preserving a chemical agent that leads to the formation in amyloid beta peptides. The inhibitors may improve neuro-psychiatric symptoms. Drugs used are-donepezil (Aricept), galantamine (Razadyne) and rivastigmine (Exelon). Second is Memantine, works on other brain cell communications. It is used in combination with Cholinesterase Inhibitors.

Culture and maintenance of animal cell line

Mammalian Cell Culture is the culture of cells that are isolated from mammalian cells from different parts of the body i.e. Skin, Liver, etc. These cells are cultured in an artificial medium. This technique is a step forward to Modern Biotechnology which ensures the cells to be preserved and further can be used for various purposes. The artificial maintenance of cells demand labour for isolation.

Cells proliferate according to their nature. Many cells grow in adherent while others grow in suspension. Mammalian cells are isolated from the multi-cell life forms, despite everything conveyed the hereditary program of inducing cell death; a procedure called "apoptosis" or "modified cell Death". It can constrain culture efficiency in biotechnological forms. Another vital issue is the limited life expectancy of essential cells; pass on after a few doublings in vitro. At the point when mammalian cells are being cultured in vitro, the specialist is endeavouring to imitate physiological condition so as to keep up and analyse the typical reactions and capacities. The way of culture medium is an imperative component of the in vitro condition.

Dulbecco's Modified Eagle Media (DMEM) is a basal medium comprising of amino acids, nutrients, glucose, salts, and a pH pointer, which contains no proteins or growth pro-motif factors. Hence, it needs supplementation to be a "Complete" medium. It is generally enhanced with 5-10% Fetal Bovine Serum (FBS). DMEM utilized a sodium bicarbonate cradle framework (3.7 g/L) and in this requires artificial concentrations of CO2 to keep up the required ph. 7-10%. The potential issue with too low CO2 level is that the pH may turn out to be excessively high. At the point when presented to encompassing dimensions of CO2, the sodium bicarbonate in the medium will cause DMEM to end up essential all around quickly. Most mammalian cell lines and essential primary cultures reproduce or grow as a thickness cell layer joined to a plastic or glass substrate. The accessible substrate surface is secured by cells, the development moderates and after that stops. Accordingly, so as to keep the cells effectively and solid developing, it is important to subculture them at intervals. More often than not, this sub development process includes breaking the securities or cell 'stick' that connects the cells to the substrate and to one another by utilizing proteolytic compounds, for example, trypsin-EDTA, dispase, or collagenase. Now and again, these chemicals or separating specialists are joined with divalent cation chelators, for example, EDTA which ties calcium and magnesium particles. The released cells are expelled from the way of life vessel, checked, weakened and subdivided into new vessels. Cells then reattach, start to develop, partition and after an appropriate brooding period (contingent upon the underlying inoculum measure, cell lines and development conditions), and again achieve confluency or immersion. Now, the sub development cycle is rehashed. As confluency of cells is reached, must be sub-cultured then other cells die. The first step in this is to detach the cells from the surface of the culture vessel by mechanical or enzymatic method. Mechanical Method involves Scrapping of cells while Enzymatic method involves the use of Trypsin-EDTA. The resultant cell suspension is meant to be split into two flasks. Now, Secondary cultures are maintained and checked periodically. Complete Media is composed of DMEM Media, FBS (Fetal Bovine Serum) and Antibiotics.

Liposomal based tranfection of animal cell line:

Transfection is an analytical method that is used in studying the function of various different genes and their products in the cells. Transfection can be done biologically, physically and chemically. The main aim is to transfer the particular Nucleic Acid to its desired subcellular region. Hence, the whole method leads to a genetically modified product. There are many approaches to perform Transfection and their use depends on the cells and the purpose of the method.

Chemical Transfection Methods are generally used because of their stability and application. They basically use Cationic Polymer, Cationic Lipid or Calcium Phosphate. Lipofectamine based Transfection is considered to be widely used method for the delivery of exogenous DNA or RNA. It is a Cationic lipid Transfection Method that forms a Liposome and hence helps in the transfer of Nucleic Acids into to the desired Intracellular Cells. The Structure of Cationic Lipid mainly consists of a +vely charged Head and 1 or 2 Hydrocarbon Chain. The head group which is charged facilitates the interaction in between the Phosphate Backbone and Lipid and helps in DNA Condensation. Hence, a liposome is formed. Charged Liposome helped in the interaction Membrane and the DNA/RNA and then facilitating the fusion of Liposome and Nucleic Acid Transfection Complex along with –vely charged

membrane of cell. The entry of this complex is facilitated by the process called Endocytosis.

When the cells are seeded i.e. reach to their 70-90% confluency, they are ready for transfection. The transfection starts with the dilution of Lipofectamine Reagent 3000 in Complete Media. Now, take your cells and add complete media along with the reagent. Incubation is given for a while. Now, the DNA-lipid complex is being formed. To confirm the Complex formation, visualize the cells under the microscope.

Cell harvesting

Cell Harvesting means the collection of the cells from the cell culture after their maintenance and to split the cells in two different flasks or culture plates to prepare more cells. Cell Harvesting can be done by various methods i.e. mechanical and enzymatic methods. Mechanical method is done by scrapping of cells while enzymatic method involves the use of Trypsin EDTA. The initial step in Cell Harvesting is detachment followed by Centrifugation. Cell Detachment is done via mechanical method by applying pressure towards one side of the flask so that cells get detached from the surface. Trypsin is a protease enzyme that is used in cell detachment which cleaves the Amino acids that helps in proving interaction with the support surface. Non-enzymatic method uses EDTA. Centrifugation helps in the collection of more dense material to settle down in the bottom. High speed can damage the cell membranes. Now, Depth filter is used to filter out the cells.

Lysis of cell by ripa lysis buffer

Lysis Buffer is a Lysis Solution that degrades the cell wall and helps in the isolation of protein from the cell. The Lysis buffer contains many components that maintain the cell. The Lysate preparation is the most common type of sample used in western blotting. The mail aim of Cell Lysis is to open up the cell to isolate various macromolecules of the cell. This is done to isolate the molecule of interest. For isolation of proteins, the desired proteins first need to be denatured. Various parameters needs to look while preparing the lysis buffer, these are pH, use of detergent, Ionic Strength, and protease Inhibitors (Thermofisher). The protein sample is then estimated through spectrophotometer.

RIPA Buffer stands for Radio Immuno Precipitation Assay Buffer. RIPA Buffer is commonly used for Western Blotting because of their specificity to proteins. Components of RIPA Buffer are following:

Tris: Tris is used in Ripa Buffer to maintain ph. Protein will precipitate or get unstable when gone out of the pH range. To avoid such problems, Tris HCl is used. This Buffer System helps in the prevention of various insoluble products along with their ions.

Sodium chloride: These Salts provide ionic strength to the buffer solution.

EDTA (Ethylenediaminetetracetic acid): EDTA acts as Metal Chelators. It is used to inhibit cation dependent proteases. EDTA basically reduces the activity of proteases and DNase Activity. It is used to dissociate proteins from other components of the cell i.e. RNA and non-ionic detergents.

Triton X-1: It acts as a non-denaturing detergent which is used to break membrane backbone structure. They are Amphipathic in nature i.e. they have both hydrophilic and hydrophobic heads to act upon. They basically separate membrane proteins from their origin i.e. Membrane. Non-ionic detergents are used in protein isolation i.e. Triton X-100. They have rigid and bulky nonpolar heads and they don't disturb the interaction and structure of water soluble proteins.

SDS (Sodium Dodecyl Sulphate): SDS is a denaturing detergent and bind to membrane and non-membrane bound proteins at a particular concentrations. The binding of SDS is cooperative i.e. if one molecule of SDS binds to a protein; it makes it easy for the other protein to bind to that SDS molecule.

Results

protease cocktail inhibitor (pci) and pmsf (phenylmethylsulfonyl flouride)

Many cells and tissues contain proteases. When Lysis buffer acts on them, they sometimes act on the target proteins. To reduce the loss of target proteins, these Protease Inhibitors are used. Many of these inhibitors require Metal ions to facilitate their function, for example, EDTA, etc (Table 1).

Table 1. *Components of RIPA lysis buffer.*

Components	Concentration (µl)
50mM Tris	25
5M NaCl	15
0.5M EDTA	1
10% Triton X-100	50
10% SDS	5
50X PCI	10
1000mM PMSF	5
Water	389

BCA i.e. Bicinchoninic Acid Method is a Protein Estimation Method. The BCA method measures the development of Cu+ from Cu2+ by the Biuret complex in soluble arrangements of protein utilizing bicinchoninic Acid (BCA). It is currently realized that there are two different responses that happen with copper particles exceptional to the BCA test. The principal response happens at lower temperatures and is the after-effect of the cooperation of copper and BCA with cysteine, cysteine, tryptophan, and tyrosine deposits in the protein. At raised temperatures, the peptide bond likewise is in charge of shading advancement. Subsequently, playing out the examine at 37◦C or 60◦C versus room temperature builds the affectability and decreases the variety in the reaction of the examine as an element of protein creation. Whenever possible, the examine

ought to be incubated at 60◦C since, after the response is finished, the absorbance does not increment considerably, while in the wake of cooling tests hatched at 37◦C to room temperature, the clear keeps on expanding in absorbance at ~2.3% each 10 min. The BCA reagent frames a complex with Cu+, which has a solid absorbance at 562 nm. BCA is profitable in that it doesn't associate with the same number of contaminants and support parts as the Folin-Ciocalteu reagent, particularly cleansers. Segments that meddle with the BCA examine either lead to decrease of Cu2+ (e.g., DTT) or copper chelators (e.g., EDTA). Total Protein Estimation is done by this method and a graph is plotted for the Standard that tells the equation for the situation. The Equation is then used to find out the concentration of the protein. The estimated concentration of the protein sample allows calculating the specific amount of it to be loaded.

In BCA Method of protein estimation, two reagents are used i.e. BCA Reagent A which contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide and BCA Reagent B which contains Cupric Sulphate, which are used in 49:1 parts.

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE is a Molecular Biology Technique used to separate Proteins and Nucleic Acid on the basis of their Molecular Mass, size and electrophoretic mobility. The separation method of macromolecules in the presence of an electric field is called Electrophoresis. The process is also known as 'Laemmli Method' after the name of Scientist U.K. Laemmli who introduced the concept in scientific studies. Western Blotting technique used two different types of gel i.e. Resolving and Stacking gel. The resolving gel basic and has high polyacrylamide concentration while stacking gel is slightly acidic and has lower concentration of polyacrylamide content. Lower concentration of polyacrylamide in stacking gel makes it porous which separates the protein poorly but allows them to form thin and sharp bands. As resolving gel's polyacrylamide concentration is high, it makes the pores smaller and hence allows then to arrange according to their molecular weight.

When these proteins loaded into the gel have –ve charge, as they are denatured because of heating; it will travel towards positive electrode. Samples are loaded along Protein ladder to identify the side of the protein. Finally, the gel is connected to electric supply. The Voltage is considered to be very important as high voltage will overheat and allows them to distort bands.

Components of sds page

Sodium Dodecyl Sulphate: Sodium Dodecyl Sulphate is particularly used to identify and isolate specific protein molecule. Proteins are amphoteric by nature that means they acquire negative and positive charge both, so SDS is used to make them negatively charged. Sodium Dodecyl Sulphate maintains a net negative charge on the molecules so that the molecules will separate according to their charge and size and strongly moves towards the Anode. The negative charge due to SDS destroys or denatures most of the proteins. SDS is an anionic detergent and along with a reducing agent breaks the disulphide bonds to small protein fragments.

Acrylamide and bis-acrylamide: Both the components are polymerised together to form a gel. The pore size of the gel through which different proteins can pass through depend upon the concentrations of acrylamide and bis-acrylamide in which acrylamide produces the linear polymers and bis-acrylamide forms the crosslinks between the chains. The minute changes between the respective concentrations can affect the electrophoretic mobility of the proteins. Acrylamide is harmful when inhaled or touched with skin, so handle with care (Table 2).

Table 2. *Concentration of Acrylamide used according to the size of the protein.*

Concentration of Acrylamide (%)	Range of Separation of Proteins
20	14702
15	16772
12.5	25842
10	15-100
8	25-100
6	40-150

Tris (of different pH): Tris is used to maintain the pH of the gel. As there are two layers of gel i.e. Resolving and Stacking Gel. Both of them need different pH for the movement of protein. The stacking gel, which has extensive pores with the goal that bigger peptides can undoubtedly move through, is normally maintained at pH 6.7– 6.8. At this pH, ionized chloride particles relocate quickly, raising the pH behind them and making a voltage angle with a zone of low conductivity, which causes glycine (from the running buffer) to ionize and move behind the chloride front. Most peptides in the example, which have a negative charge because of the bound SDS, relocate between the chloride and glycine, shaping a limited band and subsequently getting to be "stacked". When the stack achieves the resolving gel, which is at a higher pH (normally pH 8.7– 8.8), the expanded ionization of the glycine makes it quicken and overwhelm the peptides. Furthermore, the littler pore size of the settling gel begins to have a sieving impact, bringing about the partition of peptides by size. APS (APS is an oxidising agent used for the polymerisation of acrylamide and bis-acrylamide gel with TEMED.

TEMED quickens the rate of formation of free radicals from persulfate and these thus catalyse polymerization. The persulfate free radicals convert acrylamide monomers to free radicals which respond with inactivated monomers to start the polymerization chain response. The stretching polymer chains are haphazardly crosslinked by bis, bringing about a gel with a trademark porosity which relies upon the polymerization conditions and monomer focuses.

Water is used to make up the volume to the desired amount. Autoclaved Water is used (Table 3 and Figure 3).

Table 3. *Composition of 8% resolving gel and 5% stacking gel.*

Components	8% Resolving Gel (5ml)	5% Stacking Gel (1ml)
Water	2.3 ml	0.68 ml
30% acrylamide	1.3 ml	0.17 ml
1.5M Tris (pH=8.8)	1.3 ml	0.13 ml
10% SDS	0.05 ml	0.01 ml
10% APS	0.05 ml	0.01 ml
TEMED	0.003 ml	0.001 ml

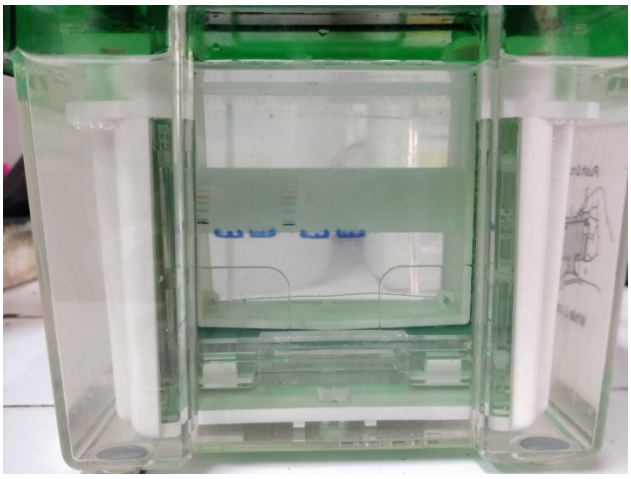

Figure 3. *Running the sample with protein ladder.*

Discussion

Protein transfer from gel on nirocellulose membrane

After the completion of Electrophoresis, the proteins need to be transferred on a membrane for further Antibody Staining followed by Detection. The transfer of protein is carried out in an Electric Field oriented perpendicular surface. Two common membranes that can be sued in Western Blotting are PVDF and Nitrocellulose Membrane. PVDF is basically used for Proteins of low Molecular Weight and provide proper mechanical support while Nitrocellulose Membrane is used because of their high ability of binding to proteins. They have additional ability to immobilise the proteins and compatibility to be used for various different Detection Methods. During the transfer of the proteins to the membrane, the gel should be placed inverted on the Black side of the cassette and a mark should be marked to identify the orientation of the samples. The Transfer takes place in Wet environment so that the membrane will be less prone to dry. During transfer, the membrane and gel are sandwiched between the sponges and filter sheet and they altogether are locked in a cassette to strongly transfer the proteins on the membrane. Two things should be kept in mind that the gel should be in close contact to the membrane and the placement of the membrane in between the positive electrode and gel. It is maintained so that the negatively charged protein can travel travels towards positively charged electrode and get transferred on the membrane. Wet and cold condition should be maintained as the current applied may dry out the gel. Hence, called as Electrophoretic Transfer [5].

Confirmation of amyloid precursor protein (APP)

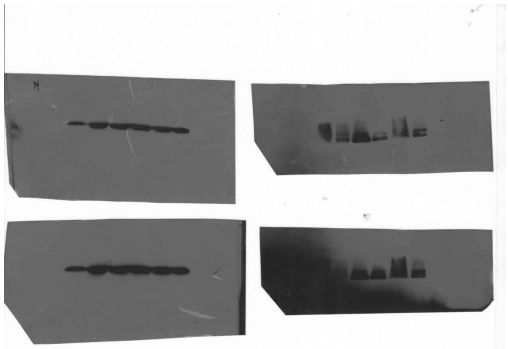

Figure 4. *APP Confirmation.*

Conclusion

Emerging technologies promise greater possibilities, such as enabling researchers to seamlessly study the profile of various Biomarkers involved in many diseases. Alzheimer's disease is now common the old age group.

To cure people from this disease, expression profile of these markers should be studied.

There are many biomarkers which are involved in amyloidgenic pathway such as, APP, BACE, BACE2, γ-secretase, C99, C83, AICD, etc. For cure and drug delivery methods, these markers can be targeted.

Drug delivery refers to the approaches or diagnostic method used to target a specific protein or gene to upregulate or down-regulate them to maintain the normal mechanisms in the body.

Many drugs should be formed that specifically target the BACE and stops it to form Aβ peptides.

Aβ can be targeted to not to form Plaques. Ultimately, the polymerisation of these peptides leads to the form of plaques. Once, these plaques stop forming, there is no formation of plaques.

Hence, there will no Neural degeneration. The advances should enable faster construction of these drugs and various gene therapy methods.

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