Variations in Hotspot Region of β-amyloid Precursor Protein (APP) Gene in Various Neurological Disorders from Hyderabad, a Cosmopolitan City of South India

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Abstract   Non-synonymous mutations/ polymorphism in amyloid precursor protein (APP) gene cause overproduction of Aβ proteins or affect its split into Aβ40 and Aβ42 peptides. Aβ42 has been considered to be a toxic peptide playing a major role in the pathogenesis of Alzheimers (AD). Similar APP plaques were observed in the brains of Down syndrome (DS) patients and high level of plasma APP was observed in patients with severe Autism spectrum Disorder (ASD). The aim of this study was to evaluate exon 16 and 17, the hotspot regions of APP gene in patients with neurobehavioral disorders like AD, DS and ASD. A total of 75 cases were recruited in the study which included AD (n=25), DS (n=25), and ASD (n=25). Polymerase chain reaction (PCR) analysis and sequencing was carried out using exon-intron encompassing primers for the selected APP gene regions. In-silico analysis was also carried out to identify the impact of sequence variants on the protein structure. Three exonic variants, two in exon 16: V683V, H684Y and one in exon 17, H733Q were identified in sporadic AD cases. Apart from these, two intronic variants were also observed. In-silico analysis showed that H733Q mutation may affect the structure and function of APP, whereas H684Y mutation is neutral. In an ASD case, our analysis showed an intronic variation ie. An A insertion at c.1964-13_1964-12insA. In-silico analysis predicted that this variation affects the elongation feature of the protein. None of the DS cases had any variation in this hotspot region. Our data indicate that variations in the selected hotspot region of APP may play an important role in the aetiology of neurobehavioral disorders.

Keywords: Alzheimer Disease, exon 16 and 17 of APP gene, Amyloid precursor protein (APP), Polymorphism, mutations

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1. Introduction

Over the past decade, there is an emerging interest in associations between neurodevelopmental and neuropsychiatric disorders. Alzheimer disease (AD) is a progressive neuropsychiatric disorder leading to the irreversible loss of neurons that slowly impairs memory and cognitive skills, which becomes severe enough to impede social or occupational functioning [1]. Senile plaques (SPs) and neurofibrillary tangles (NFTs) are considered the key pathological hallmarks of AD [2]. AD is characterized by abundant β-amyloid (Aβ) plaques in the brain, which are associated with loss of synapses, impairment of neuronal functions and loss of neurons [3]. These Aβ plaques are formed as a result of the cleavage of amyloid precursor protein (APP, OMIM 104760) into Aβ-42/Aβ-40 peptides by β and γ secretase and these errors in clevages are implicated as the pathological feature of this complex disorder.

Another common neurodevelopmental disorder is Down syndrome (DS), a chromosomal disorder, which is the result of chromosome 21 trisomy [4]. DS phenotype includes developmental abnormalities, deficiencies of the immune system, characteristic facial features, mental retardation, and congenital heart disease [5]. The brain of virtually every person with Down syndrome older than 40 years shows neurodegeneration identical to AD. Capone (2001) [6] reviewed the role of ten genes that exert an influence on central nervous system structure/function,

which might have a role in the neuropathogenesis of Down's syndrome, APP gene is included among these.

Autism/autistic spectrum disorders (ASD) are a pervasive neurodevelopmental disorder characterized by impairment in social skill and communication [7]. There is currently no single molecular marker or laboratory tool capable of diagnosing autism, however, it has been reported that plasma secreted amyloid precursor protein alpha (sAPP-α) levels were significantly increased in autistic children when compared to aged-matched controls [8].

Although there is significant aetiological heterogeneity within and between these three neurological pathologies ie., AD, DS and ASD. Recent data shows that common genetic factors like APP contribute to their aetiology. APP is a member of a family of conserved type I membrane proteins which include APL-1 in Caenorhabditis elegans, APPL in Drosophila and APP, APP like protein 1 (APLP1) and APP like protein 2 (APLP2) in mammals [9,10,11,12,13]. APP is an integral membrane glycoprotein that has an ecto-domain, a single membrane-transmembrane domain, and a short cytoplasmic tail. Full-length APP is sequentially processed by at least three proteinases α-, β- and γ-secretases (Figure 1).
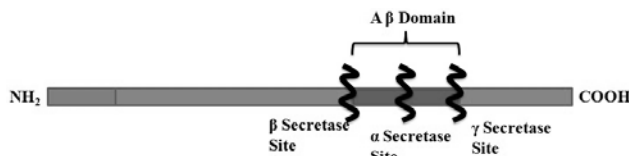

Figure 1. Proteolytic processing of Amyloid precursor protein (APP): APP is proteolytically processed in the extracellular and intracellular domains by β and then γ- secretase enzymes. The Amyloid plaques implicated in AD are formed by improper cleavage of the APP protein.

George-Hyslop et al. [14] identified a genetic defect causing autosomal-dominant Alzheimer's disease on the long arm of chromosome 21, which was subsequently identified to be the locus of APP gene. The first pathogenic mutation in APP was reported by Levy et al. [15] Various other pathogenic mutations in this gene have been identified over the years, [16] interestingly; most of these were located in exons 16 and 17, which encode the Aβ region of APP, hence known as the hotspot mutations. Sequence changes in these regions affect the protein's native structure as there is a failure to recognize cleavage sites which result in altered proteolytic processing of APP. The present study was an effort to identify mutations in the hotspot regions of APP ie., exon 16 and 17 in sporadic AD, DS and ASD subjects from Hyderabad a cosmopolitan city of South India and establish their functional significance using different in-silico methods.

2. Methods

2.1. Sampling

A total of seventy five patients were included in this study, twenty five AD patients were clinically identified based on the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorder Association for probable AD (NINCDS-ADRDA) [17] and were considered eligible for inclusion in the study during the period 2010 to 2014. Cytogenetically confirmed twenty five children

with DS and another twenty five children with ASD were included based on Indian Scale for Autism Assessment (ISAA) which is an Indian assessment tool based on the criteria laid down in "Diagnostic & Statistical Manual of Mental Disorders-IV" and Childhood Autism Rating Scale (CARS). The study protocol was approved by the Institutional Ethics Committee. From each patient/attendee a detailed clinical and family history along with 2ml of blood sample was collected in an EDTA vacutainer. Informed Consent was taken from caretakers/guardians of the patients.

2.2. Mutational Analysis

DNA was isolated following the salting-out method routinely used in our laboratory [18]. APP exon 16 and exon 17 were analysed for all 75 patients by PCR. Specific set of primers were used to amplify the two regions as mentioned in Table 1 [19,20] PCR was carried out as described earlier by our group [21]. Briefly, 94°C, 4 min to denature; then 35 cycles of 94°C, 30 sec, 57°C, 30 sec; 72°C, 45 sec and 72°C, 5 mins for final extension. Amplified PCR products were run on a 2 % agarose gel, and their band images were analyzed with UV I Tech gel documentation system (Cambridge, UK).

Table 1. Primers used to amplify the exon 16 and exon 17 regions

Exon	Primers
Exon 16	F: 5`-GGGTAGGCTTTGTCTTACAG-3` R: 5`-GGCAAGACAAACAGTAGTGG-3`
Exon 17	F: 5'-GACCAACCAGTTGGGCAGAG-3' R: 5'-CATGGAAGCACACTGATTCG-3'

Amplicons of exons 16 and 17 were purified using the QIAquick (Qiagen) gel extraction kit, followed by bidirectional automated sequencing reaction using the BigDye v3 termination chemistry (Applied Biosystems, Foster City, CA) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The sequence chromatograms were analyzed using Chromas 2.21 software (Technelysium Pty Ltd, Queensland, Australia).

2.3. In Silico Analysis

In silico analysis was carried out using five different programmes described below:
(i) Sorting Intolerant From Tolerant (SIFT) program can distinguish the functionally neutral and deleterious amino acid changes; hence, it is widely used to detect if an amino acid substitution affects the structure and function of a protein molecule (http://blocks.fhcrc.org/sift/SIFT.html) [22].
Classification of SIFT scores is as follows: Deleterious=.00-0.09, Tolerant= 0.10-1.00.
(ii) PolyPhen-2 (Polymorphism Phenotyping v2) tool determines the position and impact of an amino acid substitution on the structure and function of human protein basing on combinational information of phylogenetic, structural, and sequence annotations. It receives amino acid sequence or corresponding Swiss-Prot ID and position of mutant as input data [23]. PolyPhen-2 algorithm calculates the naïve Bayes posterior probability that a given mutation will be damaging and qualitatively predicts that it will be benign, possibly damaging, or probably damaging, corresponding to posterior probability intervals (0 - 0.2), (0.2 - 0.85), and (0.85 - 1), respectively.

(iii) The SNPs3D algorithm bases on support vector machine (SVM) technique to determine whether nonsynonymous SNP is deleterious or nondeleterious to that of protein function. In principle, it employs two methods:

1) First method uses the protein structure to identify which amino acid substitution significantly destabilizes the folded state

2) Second method analyzes the extent and nature of amino acid conservation in the affected sequence position to identify deleterious substitutions [24].

A negative value indicates that the mutated protein is deleterious and vice versa.

iv) MutPred is a web application developed to classify an amino acid substitution (AAS) as disease-associated or neutral in humans. In addition, it predicts molecular cause of disease/deleterious AAS [25]. It indirectly exploits the structural and functional data available for functional prediction, effectively enlarging the training data sets beyond the characterized disease causing events. The output of MutPred contains a general score (g), which defines the probability of amino acid substitution to disease-associated, and top five property scores (p), where p is the P-value on which certain structural and functional properties are impacted. Scores with $g > 0.5$ and $p < 0.05$ are referred to as actionable hypotheses while scores with $g > 0.75$ and $p < 0.05$ are referred to as confident hypotheses and scores with $g > 0.75$ and $p < 0.01$ are referred to as confident hypotheses.

v) Variant Effect Predictor, The VEP was formerly known as the SNP Effect Predictor, determines the effect of your variants (SNPs, insertions, deletions, CNVs or structural variants) on genes, transcripts, and protein sequence, as well as regulatory regions [26].

3. Results

3.1. Clinical Analysis

AD subjects included in the study were in the age range of 57 to 87 years with a mean age at onset of symptoms was 68.9±9.23 years. 57% of the patients were males and 43 % were females. AD subjects were categorized into three groups based on the extent of cognitive impairment 19% having mild, 52% having moderate and 29% having severe cognitive impairment. The AD patients also had other comorbidities like diabetes (57%), hypertension (23%), hypothyroidism (4.7%) and coronary artery disease (19%). 1.3 % of the patients had a combination of the above mentioned comorbties. None of their families had any history of dementia and related disorders.

Of the 25 children with DS 72% were males and 28 % were females, all in the age range of 3 to 41 years with a mean age of 10.84 ± 8.62 yrs. They were categorized into three groups based on the extent of mental retardation 68% having mild, 28% having moderate and 4% having severe mental retardation. Cardiac defects (12%) and leukaemia (4%) were reported in some DS patients.

The children with ASD were in the age range of 3 to 13 years with a mean age of 12.05 ± 8.82 yrs. 81% were males and 19 % were females, 4 % of them had a positive family history of ASD. Individuals with ASD were categorized into two groups based on their behavioural activity 71% were showing hyper active behaviour while 29% had hypo/ and mellow behaviour.

3.2. APP Mutation Analysis

Two PCR products per patient were obtained, one for exon 16 and another for exon 17 and their flanking introns of of APP gene. Sequences were obtained for all 150 amplicons (50 each from AD, DS and ASD subjects) and were analysed for reported and novel sequence variations using NCBI- BLAST tool and nucleotide numbering was according to the Cell surface protein APP sequence (GenBank accession number NM_000484), with the A of ATG initiator codon as nucleotide position one. Four individuals (ALZ12, ALZ 16, ALZ13 and ALZ9) with AD showed sequence variations. Patient ALZ12 with a double mutation in exon 16 at c.2049T>C (V683V) and c.2050 C>T (H684Y) and another patient ALZ16 showed a variation in exon 17, c.2199 T>G leading to a H733Q amino acid change (Table 2).

Table 2. Mutations identified in hotspot regions of APP gene in AD and ASD subjects

S.NO.	CODE	AGE/SEX	SEVERITY OF DISORDER	AGE OF ON-SET	REGION IN APP	VARIATION	CODON CHANGE	AMINO ACID CHANGE
ALZHEIMER DISEASE (AD)								
1	ALZ 12	66 Yrs./M	Moderate	63 yrs.	exon 16	c.2049T>C	GTT to GTC	p.V683V
						c.2050 C>T	CAT to TAT	p.H684Y
2	ALZ 16	60 Yrs./F	Moderate	56 yrs.	exon 17	c.2199 T>G	CAT to CAG	p.H733Q
3	ALZ 13	69 Yrs./M	Moderate	66 yrs.	flanking exon 16 (intron 15)	c.1964-78G>T	---	---
4	ALZ 9	88Yrs/M	Severe	86 yrs.	flanking exon 17 (intron 17)	c.2211+58_2211+59insT	---	---
AUTISM SPECTRUM DISORDER (ASD)								
5	ASD 15	12Yrs/M	Hyperactive behaviour	--	flanking exon 16 (intron 15)	c.1964-13_1964-12insA	---	----

Two intronic variations, c.1964-78G>T in intron 15 (earlier reported as SNP rs112182125, c.1964-78G>C) and T insertion at c.2211+58_2211+59insT in intron 17 an unreported variation (Figure 2) were identified in patients ALZ 13 and ALZ, respectively.

Of the 25 DS children analysed by sequencing, none showed any sequence variation in the selected regions while, our analysis revealed an intronic variation at c.1964-13_1964-12insA (Figure 3) in one of the ASD patient (ASD 15).
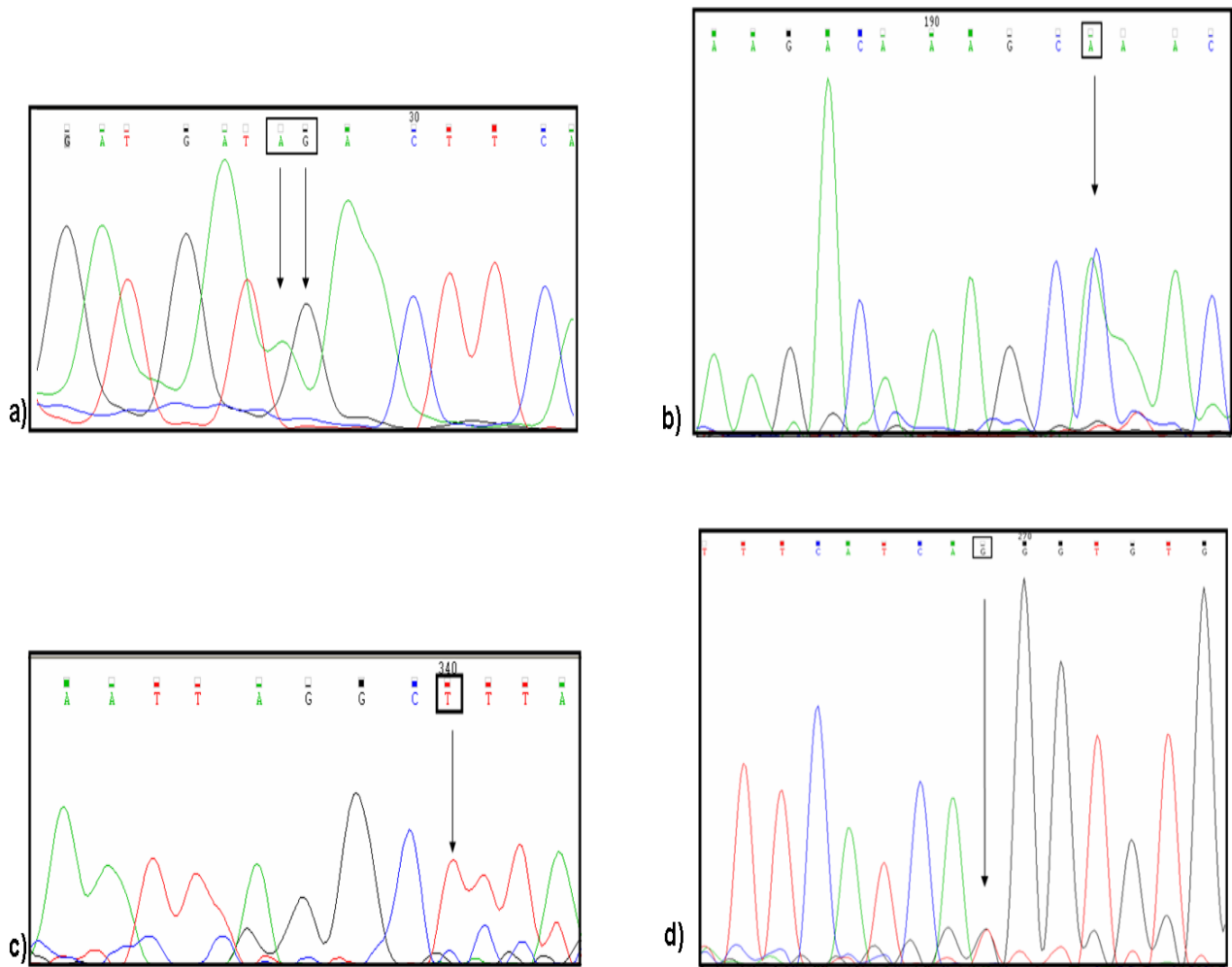
Figure 2. –Variations found in AD patients: a) c.2049T>C, c.2050 C>T b) c.1964-78G>T c) c.2199 T>G/T d) g.284157 _284158insT sequence variants found in exon 16, intron 15, exon 17 and intron 17 respectively
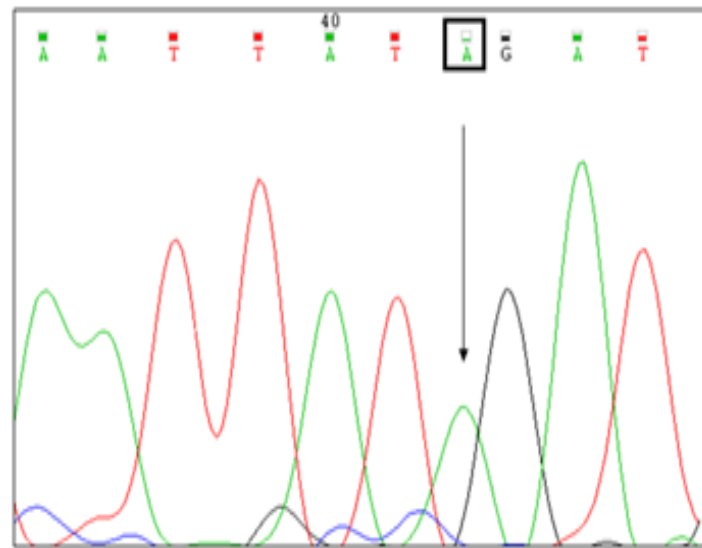

Figure 3. – Variations found in ASD subject: c.1964-13_1964-12insA found in the intron15

3.3. In Silico Analysis of APP Variants:

The results of different types of in silico testing of the missense mutations and other intronic variations identified in APP exons are given in Table 3. We have used various in silico tools like SIFT, Polyphen, SNPs3D and MutPred (details in materials and methodology) to predict the effect of the variation on the structure function and of resulting protein product. Variant Effect Predictor (VEP) was also used to predict the effect of intronic variations on the protein function. According to the in-silico analysis c.2050 C>T change was showing no effect on the structure and function of the APP protein. On functional analysis, the identified sequence change in exon 17 (c.2199 T>G) was predicted as damaging.

Table 3. Prediction results of sequence variations by different algorithms in AD

Genomic position	cDNA position	Amino acid change	Exon	Clinical significance
Chr 21:27269899 G>C(hom)	c.2050 C>T	p.H684Y	16	PolyPhen= benign(0.078); SIFT= tolerated(0.96) SNPs3D= 1.87 MutPred= 0.587
Chr 21:27264046 A>C/A (het)	c.2199 T>G	p. H733Q	17	PolyPhen=probably_damaging(1); SIFT=deleterious(0) SNPs3D= -0.85 MutPred= 0.622
* Range and interpretation SIFT Scores: Tolerant= 0.10-1.00, Deleterious=.00-0.09 PolyPhen-2 Scores = benign =0 - 0.2, possibly damaging = 0.2 - 0.85, probably damaging = 0.85 – 1 SNP3D Scores= -ve= deleterious substitution, +ve= non deleterious substitutions MutPred Scores= MutPred score > 0.5 could be considered as 'harmful', while a MutPred score > 0.75 should be considered a high confidence 'harmful' prediction.				

Table 4. Prediction results of sequence variations by Variant Effect Predictor in ASD

Genomic position	Region in APP	Variation	Consequence	Effect
Chr 21:27269997_27269996 ins A	Intron 15	c.1964-13_1964-12insA	intron_variant, feature_elongation	This sequence variant causes the extension of the genomic feature with regard to the reference sequence

4. Discussion

Several candidate gene and genome-wide association studies (GWAS), in recent years have increased the knowledge regarding the genetics of neurobehavioral disorders especially AD [27,28,29,30,31]. The physiological role and processing of APP appears to be important in the aetiology of AD [32]. A series of different mutations were discovered in the APP gene in patients with familial AD. Currently, there are about 30 known coding mutations in APP gene and 25 of them are reported as pathogenic resulting in early onset AD. [33] Most of the known mutations identified in APP gene were primarily clustered around the secretase processing sites, hence called hotspot region [32]. Mutations at or near the β- and γ- proteolytic sites present in the Aβ domain appear to result in over production of either total amyloid- β or shift in the Aβ 40: Aβ 42 ratio towards formation of the more toxic Aβ 42 peptide, whereas substitution within the amyloid-β peptide are believed to result in formation of amyloid-β plaques with increased propensity for aggregation [34,35] which is the hallmark of AD and other neurodevelopmental pathologies.

The histopathological changes characteristics of AD brain are also observed in persons with DS, especially beyond third decade of life [36]. It is well established that iindividuals with DS are much more likely to develop dementia of AD (DAD) than the general population [37]. Similar Aβ plaques were also found in postmortem brains from individuals with autism [38] indicating that the defect in APP may play a crucial role in ASD.

Since most of the reported APP gene mutations were found in a hotspot region including exon 16 and 17 [39,40] we evaluated this region by PCR-Sequencing. To the best of our knowledge this region has not be investigated in AD, DS and ASD patients from India. Our study was the first to identify both known and novel variations within exons 16, 17 and their flanking regions of APP gene. We identified APP sequence variations in four cases of AD and one patient with ASD. None of the DS cases showed any sequence variation in these regions indicating that APP gene and possibly some of its neighbouring genes, when amplified, cause neurodegeneration as indicated by

Rovelet-Lacrux et al (2006) who reported duplication of the APP Locus on chromosome 21 in 5 families with autosomal dominant early onset Alzheimer disease (EOAD) and cerebral amyloid angiopathy [41].

In ALZ 12 patient with AD, APP exon 16 exhibited a double mutation at c.2049T>C / c.2050 C>T. One was a silent mutation while the other was a missense mutation with H684Y aminoacid change. According to the in-silico analysis c.2050 C>T change was showing no effect on the structure and function of the APP protein.

Variation in APP exon 17, c.2199 T>G was reported in ALZ 16 patient with AD with an early onset symptoms (56years) and the amino acid variation was H733Q change. This change was predicted as damaging and further MutPred tool predicted that c.2199 T>G change causes the loss of a catalytic residue which may lead to lowering the functional efficiency of the protein. In an earlier study, H733P c.2198 A>C mutation was detected in a healthy individual from the Mandenka population, hence was non-pathogenic mutation [42]. In our study, Histidine to Glutamine change at the same site was predicted as pathogenic and disease related. Liu et al (1999) stated that substitution of His-13 residue is responsible for the different Zn2+-induced aggregation pattern of rat and human Aβ [43]. Since Histidine is the only amino acid with an imidizole in side chain which makes it charged and basic, predicted pathogenicity of H733Q in our study, can be as a result of the substitution of charged to uncharged amino acid, which may be the reason for the predicted loss of catalytic residue. The in-silico results therefore indicate that c.2050 C>T mutation in exon 16 is less damaging to the structure of APP, compared to c.2199 T>G mutation in exon 17.

Two intronic variations were also observed, one at c.1964-78G>T in intron 15 which is a known polymorphism (rs112182125, c.1964-78G>C) in ALZ 13 patient and another variation in intron 17, a T insertion at c.2211+58__2211+59insT in ALZ 9 patient. The functions of these are unclear and neither of these variations lie at the splice site.

A patient diagnosed with ASD (ASD15), had a c.1964-13_1964-12insA. The patient was 12 years old with an aggressive behaviour and severe mental retardation. In-silico analysis indicated that the variation affects the elongation feature of the protein. This variation can cause

change in least one base of the terminator codon (stop codon) resulting in an elongated transcript or frame shift of the reading frame that causes the translational reading frame to be extended relative to the reference feature.

Apart from the pathogenic mutations, AD protective mutation like A673T mutation in APP was also analysed. This mutation was found by Jonsson et al in Icelander population [44]. This mutation was found to be protective against AD and age related cognitive decline. Our analyses indicated that none of the patients showed this mutation suggesting indicating an increased β-cleavage of APP which is a risk for the disease. Tanzi et al [45] who sequenced exons 16 and 17 of APP gene in Familial and sporadic AD cases, found no mutations in either groups and suggested that APP gene mutations may account for only some cases of Alzheimer's disease and other regions of APP also needs to be evaluated.

5. Conclusion

We have identified two novel variants one in exon 16 and the other in exon 17 of APP gene along with two intronic polymorphisms in AD subjects. Of the two exonic variants, c.2199T>G appears to be pathogenic. An A insertion in the inronic region of ASD subject also revealed its effect on the protein translation predicting a formation of abnormal protein. Our data indicate that variations in the selected hotspot region of APP may play an important role in the aetiology of sporadic AD; Presence of a variation in the hotspot region of APP gene seen in patient with Autism indicated the probable role of APP in this disorder.

Acknowledgement

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