APP Functional Mutation

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Student Declaration:

I declare that all the work presented in this dissertation is my own, except where otherwise stated.

Signature:

Date:
Abstract

A novel deletion of 6 bp was found in intron 17 of APP gene. Bio-informatics tools predict that this mutation could be altering splicing pattern since it overlaps with potential branch points, enhancer motifs, potential acceptors and donators of splice sites. Minigene splicing analyses in COS-7 and BE(2)-C cells were performed in order to identify changes in the expression of exon 17. The results showed that intron 16 and 17 were correctly removed while exon 17 was retained in both cell lines. This could be inferring several things:

a) The mutation is not protecting the patient from AD due to its location in an intronic region.
b) The efficiency of bio-informatic tools prediction of the impact of the deletion in degenerated positions was low and inaccurate.
c) The mutation could be interfering with the isoform ratio production. That is if the deletion is part of splicing motifs, trans-acting splicing elements could be delayed at the moment of motif recognition. This could in turn be leading to an alteration in the rate of the splicing process and therefore protein production.

Further assays like RNA extraction from patient’s brain tissue together with real time PCR may help to elucidate if this 6 bp mutation plays a protective role in AD.
1. Introduction

Alzheimer's disease (AD) is the most common form of pre-senile and senile dementia. Its incidence rises with age (1). It begins with subtle memory failure, which progresses to a more severe form that is eventually incapacitating (2-4). Other symptoms include confusion, poor judgment, language disturbance, hallucinations, incontinence, mutism, etc. The clinical duration of the disease is eight to ten years (2, 5). Cerebral cortical atrophy seen in AD is a product of intraneural neurofibrillary tangles and large extracellular accumulations of amyloid-β (Aβ) in the form of senile plaques and cerebrovascular deposits. These Aβ aggregates are thought to trigger an inflammatory response, neuronal cell death and gradual cognitive decline (1, 3, 4, 6-10).

Genetically, AD is considered a polygenic and multifactorial disorder (1, 5). AD displays no single or simple mode of inheritance. However, genetic studies identified that all cases of familial AD are linked to genes that affect the processing of Aβ. Rare, early-onset autosomal dominant and fully penetrant forms are caused by mutations in three genes: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2; Table 1). By contrast, late-onset or sporadic AD seems to be governed by an array of common risk alleles across a number of different genes. At least one genetic polymorphism had been identified as a risk factor, the ε4 allele of the apolipoprotein E gene (APOE) that is believed to interact with APP and Aβ (4, 9, 11-14). Late or early onset and familial or non-familial AD have the same pathology and clinical phenotype. Thus, the only way to discriminate them is according to family history and molecular testing (5, 11, 15).

Table 1. The relative proportion of each subtype of Alzheimer's Disease and the related genes. Modified from Pagon et al 1993-2014.

<table>
<thead>
<tr>
<th>CAUSE</th>
<th>% CASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal (Down syndrome)</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>All familial</td>
<td>~25%</td>
</tr>
<tr>
<td>• Late-onset familial &gt; 65 years old</td>
<td>15%-25%</td>
</tr>
<tr>
<td>• Early-onset familial AD &lt; 65 years old:</td>
<td></td>
</tr>
<tr>
<td>- APP</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>- PSEN1</td>
<td>10%-15%</td>
</tr>
<tr>
<td>- PSEN2</td>
<td>20%-70%</td>
</tr>
<tr>
<td>Unknown (includes genetic/environment interactions)</td>
<td>~75%</td>
</tr>
</tbody>
</table>
The accumulation of neurotoxic amyloid (Aβ) derived from the post-translational proteolysis of APP in the central nervous system appears to represent a major pathological step in the progression of AD (7, 15).

1.1. Amyloid precursor protein (APP)

APP gene contains 19 exons encoding a large precursor protein of 695-770 amino acids and is located within the region 21q11.2-q21.05 (16, 17). So far, in AD & FTD Mutation Database, 40 mutations had been documented in APP gene mainly in exons 16 and 17 where the Aβ peptide is encoded. APP mutations have been detected in a few families worldwide. The most common mutation results in a valine to isoleucine substitution at codon 717. It is thought that the pathogenic mutations alter the metabolism of APP increasing the production of Aβ peptide (Table 2) (1, 3, 18). Aβ is a normal soluble cellular metabolite comprising two predominant forms with different COOH-termini Aβ40 and Aβ42. It has been proposed that an increasing ratio of Aβ42 versus clearance of Aβ leads to a slow rise in its steady state levels in brain tissue causing AD. Since it has been proposed that Aβ42 is more amyloidogenic and forms part of the core of the amyloid plaques (2, 4, 7, 8, 19). APP undergoes posttranslational proteolytic processing by alpha, beta, and gamma-secretases. Alpha-secretase generates soluble amyloid protein, while beta- and gamma-secretases generate APP components with amyloidogenic features. These two processing pathways are mutually exclusive (10, 11, 15, 20). While there are mutations that markedly increase the production of Aβ peptide, there is also protective mutation against AD like A673T substitution in APP. Aspartyl protease β-site APP cleaving enzyme (BACE1), cleaves the carboxy-terminal fragment at sites, with preference for positions 40 and 42, leading to formation of amyloid Aβ40 and Aβ42 peptides. A673T proximity to the proteolytic site of BACE1 result in impaired cleavage of APP. Carriers of A673T present a reduced production of Aβ compared to wild type APP (21).

Table 2. Most common APP pathogenic variants.

<table>
<thead>
<tr>
<th>DNA Nucleotide Change</th>
<th>Protein Amino Acid Change</th>
<th>Relevance to AD pathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.2149G&gt;A</td>
<td>p.Val717Ile</td>
<td>Increase in Aβ production or Aβ42/Aβ40 ratio; mutations in the Aβ sequence or close to the β- and γ-secretase site of APP; locus duplications</td>
</tr>
<tr>
<td>c.2010_2011delinsTC</td>
<td>p.Lys670_Met671delinsAsnLeu</td>
<td></td>
</tr>
<tr>
<td>c.2075C&gt;G</td>
<td>p.Ala692Gly</td>
<td></td>
</tr>
<tr>
<td>c.2018C&gt;T</td>
<td>p.Ala673Val</td>
<td></td>
</tr>
<tr>
<td>c.2078A&gt;G¹</td>
<td>p.Glu693Gly¹</td>
<td></td>
</tr>
</tbody>
</table>

Every year thousand of mutations are identified either affecting protein expression or mRNA splicing. Variations in sequences produced by synonymous, non-synonymous or non-sense mutations can create or disrupt splice sites or auxiliary cis-splicing sequences leading the appearance of truncated proteins or the lack of the correct gene product (22). Alternative splicing is a ubiquitous mechanism for controlling protein expression which occurs as part of the pre-mRNA maturation process in each eukaryotic cell (22, 23). More than 60% of mammalian pre-mRNA is alternative spliced which is widely prevalent in the nervous system (23). This event is governed by a macromolecular machine called spliceosome, consisting of five small nuclear RNA (snRNA) molecules (U1, U2, U4, U5 and U6) and other 150 polypeptide (22, 23). A coordinated assembly of these 5 molecules with small nuclear ribonucleoproteins particles (snRNP) results in the removal of each intron and the ligation of the flanking exons. Alternative splicing is guided by multiple exonic and intronic cis-elements and trans-acting splicing factors. For instance, Exonic Splicing Enhancer (ESE) is a specific short nucleotide sequence targeted by Serine/Argine-rich (SR) proteins, which then promote exon definition. Conversely, the Exonic Splicing Silencer (ESS) prevents the spliceosome recognising pseudo exons and decoy splice sites. There are also intronic elements with similar function, Intronic Splicing Enhancer (ISE) and Intronic Splicing Silencer (ISS) (23). Splice patterns vary according to tissue location, stage of development and so on, synthesizing different proteins from the same gene (22).

APP has at least four isoforms generated by alternative splicing of exons 1-13, 13a, and 14-18. The predominant transcripts are APP695 (exons 1-6, 9-18, not 13a), APP751 (exons 1-7, 9-18, not 13a), and APP770 (exons 1-18, not 13a). All of these transcripts participate widely in adhesion, neurotrophic and neuroproliferative activity, intracellular communication and membrane-to-nucleus signaling (9, 24, 25). APP is expressed almost ubiquitously throughout the body, with different isoform ratios in specific tissue types (9). These isoforms differ in that APP751 and APP770 contain exon 7, which encodes a serine protease inhibitor domain. APP695 is the predominant form in neuronal tissue, whereas APP751 is the predominant variant elsewhere (7, 15, 25).

Previous sequencing efforts by our lab (unpublished) found a novel heterozygous 6 bp deletion (IVS17-83 ΔAAGTAT) in sample Oxford 0483 belonging to a male patient with early onset Alzheimer’s disease (EOAD) and no family history of cognitive impairment. This mutation is located on intron 17 of the APP gene. Human Splicing Finder analysis predicted that it overlaps with potential branch points, enhancer motifs and potential acceptors and donators of splice sites. One particular site showed 100% disruption of a 6 bp enhancer site (IVS17-82) predicted to bind SRp55 (SRSF6). These enhancers are recognized by Serine/arginine-rich Splicing Factor (SRSF6), which are highly conserved in Eukaryotes and
essential for both constitutive and alternative splicing. SR proteins contain one or two RNA recognition motifs at the N-terminus, which determines RNA binding specificity, and at the C-terminus an arginine-serine rich domain that promotes protein-protein interactions within the splicing complex. During splicing SR proteins function in modulating the 5' and 3' splice sites in a concentration manner. This protein plays a role in several mechanisms of regulation in various genes like Microtubule-Associated Protein Tau (MAPT), which in adult human brains produces equal amounts of exon 10+ and exon 10−. It has been documented that intronic mutations (e.g. deletions) result in the alterations in the ratio of 10+/10− isoforms leading to Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (23).

Besides disrupting a constitutive splice site, other mutations can be activating cryptic splice sites that will generate competition for constitutive site recognition by the splicing machinery or the inclusion of a pseudo exon. Since these sites are localized in introns at short or long distance from the constitutive splice sites, they are usually harder to predict the outcome (22).

Exon 17 is always retained in the 3 isoforms that this protein presents. Its removal would mean an impairment of this protein. This project aims to identify if this 6 bp deletion could be causing exon 17 removal, due to cryptic or pseudo exons normally are activated by intronic mutations (26). Thus, this deletion could be altering the ratio of APP isoform production. In this case, a decrease in the production of Aβ could be having a protective effect from AD (27). Besides, as the patient was diagnosed with AD this could also be suggesting that there are other major genetic players in the development of AD apart from those which are intervening in the amyloid cascade.

2. Materials and Methods

A 506 bp fragment from APP gene was amplified by PCR using a heterozygote DNA sample and the primers Splice E17F and Splice E17R (Table 3). The sequence amplified included 167 bp of intron 16, exon 17 and 192 bp of intron 17. The PCR product was used in a ligation reaction with the vector pCR™4-TOPO from Invitrogen TOPO TA Cloning.
<table>
<thead>
<tr>
<th>Process</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>APP fragment amplification</strong></td>
<td><strong>Primer Forward</strong></td>
</tr>
<tr>
<td></td>
<td>Splice E17F: 5’ CAA ATA GTC GAC CAA CCA GTT GGG CAG AGA AT 3’</td>
</tr>
<tr>
<td><strong>Sequencing</strong></td>
<td>M13 F: 5’ GTA AAA CGA CGG CCA G 3’</td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td>pET01S: 5’ GAT CGA TCC GCT TCC TG 3’</td>
</tr>
</tbody>
</table>

**2.1. Transformation of bacteria with plasmid DNA**

The transformation reaction contained 1 µl of the ligation mixture and 50 µl of One Shot® TOP10 Competent cells (Invitrogen), which was incubated on ice for 15 minutes. The transformation mixture was heat-shocked at 42°C for 30 seconds. Cells were incubated on ice for 2 minutes. After, 250 µl of SOC Medium (Invitrogen) was added and followed by 1 hour of agitation at 37°C. Then, 20 µl and 100 µl of the transformation mixture were spread into plates containing 0.004 g/ml Circlegrow (MP Biomedicals), 0.015 g of Agar (Invitrogen), 0.05 mg/ml Ampicillin (Fisher) and 40 µl of 4% X-gal (Fisher). Finally, plates were incubated at 37°C for 16-18 hours.

**2.2. Screening process**

Plates were covered with white and blue colonies. The identification of the colonies containing the insert was based on their white color, since only transformed cells are unable to process X-gal and due to this they remained in their white natural color. The DNA sample used originally in the PCR was heterozygote. Therefore, some white colonies will contain the wild type plasmid and other white colonies will have the mutant plasmid containing the 6 bp deletion. DNA extraction was carried out using QIAgen QIAprep Spin Miniprep Kit following manufacturer’s protocol. The DNA was checked for its quality and quantity with NanoDrop 3300 (Thermo Scientific).

**2.3. DNA sequencing**

The sequencing was performed using the Applied Biosystems® BigDye Terminator V3.1. cycle sequencing Kit. Each reaction contained a mixture of 150-300 ng/µl of DNA, 1 X
Sequencing Buffer, 5 pmol of primer M13F or M13R (Table 3), 2 µl of BigDye and H₂O to a final volume of 10 µl. The sequencing protocol consisted of 25 cycles of an initial temperature of 96°C for 30 sec, followed by 50°C for 15 sec and a final temperature of 60°C for 4 min. The samples were placed into Performa DTR Gel filtration Cartridges (Edge Biosystems). The tubes were centrifuged for 3 min at 2350 x g. The supernatant was placed in a new 0.5 ml tube. Finally, the samples were incubated at 90°C until completely dried. The sequencing was then analysed on an ABI 313A automated sequencer.

2.4. Recombinant plasmid digestion

Both mutant and wild type TA clones were digested in order to isolate the insert. Each reaction contained 1000 ng/µl of TA clone, 1 X of Digestion Buffer (Fermentas), 0.1 µ/µg of SalI (Fermentas), 0.1 µ/µg of XbaI (Fermentas) and nuclease-free distilled H₂O, which was added to a volume of 20 µl. Next, 5000 ng of the minigene vector (pETO1) was digested in a reaction that includes 1 X of Digestion Buffer, 0.1 µ/µg SalI, 0.1 µ/µg of XbaI, 2 µl of Fast AP (alkaline phosphatase; Fermentas) and nuclease-free distilled H₂O to a final volume of 50 µl. All digest reactions were incubated at 37°C for 30 min, following by a period of 20 min at 65°C.

2.5. DNA extraction from gel

All digest reactions were loaded on a 1% Agarose gel. The expected digested bands were cut and DNA was extracted by QIAquick Gel extraction Kit (QIAGEN). Manufacturer’s protocol was followed.

2.6. Ligation

The amount of insert needed for each ligation reaction was calculated using the following equation.

\[
\text{ng insert} = \left( \frac{100 \text{ ng pETO1 insert size (bp)}}{\text{vector size (bp)}} \right) \times 3
\]

T4 DNA ligase kit (Invitrogen) was used following manufacturer’s protocol.

2.7. High Efficiency Transformation

The transformation was carried out using high efficiency NEB 5-α Competent E. coli cells following manufacturer’s protocol.
2.8. DNA Plasmid extraction

A total of 18 colonies were selected for insert screening using the Promega PureYield Plasmid Miniprep System kit following the manufacturer’s protocol. Digested reactions with SalI and XbaI were set-up to verify that pET01 contained the insert.

2.9. Sequencing

The pET01 vectors containing the mutant or wild type insert were sequenced. The reaction was performed using the same conditions described in section 2.3. However, this time the primers were pET01S and pET01AS (Eurogentec; Table 3).

2.10. Endotoxin free plasmid DNA purification

Macherey-Nagel, NucleoBond® Xtra Midi EF/Maxi EF kit was used for an ultra fast purification free of endotoxins of the pET01 plasmid. The protocol was followed according to the manufacturer’s instructions.

2.11. Cell culture

COS-7 cells are monkey African green kidney cells and BE(2)-C Human neuroblastoma astrocytoma cells; both of them were obtained from the European Collection of Cell Cultures (ECACC). COS-7 were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma) with 10% FBS (Fetal Bovine Serum; Gibco), 2 mM L-Glutamine, 100 U/ml penicillin (Gibco), 100 µg/ml of streptomycin (Gibco) and 2.5 µg/ml fungizone to prevent bacterial and fungal infection.

BE(2)-C were cultured with half of Eagle’s Minimum Essential Medium (EMEM; Sigma) and half of Ham’s F12 supplemented with 1% of non-essential amino acids, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 10% FBS, 100 U/ml penicillin, 100 µg/ml of streptomycin and 100 U/ml fungizone.

The media was filtered through a 0.5 µm filter, stored at 4°C, and warmed to 37°C before use. Cells were grown in 75 cm² culture flasks in an incubator at 37°C with humidified atmosphere containing 5% carbon dioxide. Cells were split when they reached a confluence between 90-100%. Old medium was removed and cells were washed with PBS. Cells were detached from the bottom of the flask by trypsinisation using 3 ml of trypsin-EDTA solution and incubated at 37°C for 2-5 minutes. To stop trypsin action, 3 ml of complete medium was
added. An appropriate volume was transferred to a new flask and the volume made up to 15 ml with complete medium.

2.12. Cell counting

The cells were trypsinised by the same process described above and counted using a hemocytometer. Then, $3.5 \times 10^5$ COS-7 cells or $6 \times 10^5$ BE(2)-C cells and 5 ml of medium were added to each 6 mm culture dish and incubated at 37°C.

2.13. COS-7 and BE(2)-C Cells transfection

Cells were transfected when they were between 50-80% confluent. The experiment was repeated three times in triplicate with each cell line. The transfection mixture contained 2 ml of DMEM or EMEM +F12 without any additives, 1000 ng/µl of DNA and 9 µl of Transfast (cationic liposomes design to deliver nucleic acids into the cells; Promega). The mixture was vortexed and incubated for 15 min. Then, old medium from the cells was removed and 4 ml of PBS was used to wash the cells. The mixture was added to the cells and incubated for 1 hour. Then, 4 ml of complete medium was added and the cells were incubated for 24 hours.

2.14. RNA extraction

Cells were harvest by trypsinisation (refer to section 2.11.) and centrifuged for 5 min at 2350 g. RNaseasy mini kit (Qiagen) was used to extract the total RNA and the manufacture’s protocol was followed.

2.15. cDNA synthesis

RNA was treated with DNase (Ambion® TURBO DNA-free Kit). Synthesis of cDNA was done using Agilent Technologies AffinityScript Multi Temperature cDNA Synthesis Kit following the manufacturer’s protocol. Next, 2000 ng/µl of RNA was used for each reaction. cDNA was obtained using Random primers or Oligo (dT). Reactions containing only RNA, primers, buffer, dNTPs but not enzymes were included as negative controls to detect any residual DNA from the RNA extraction.

2.16. Polymerase Chain Reaction

Each 30 µl amplification reaction contained 1 X of PCR reaction Buffer + 1.5 mM MgCl$_2$ (Roche), 0.2 mM of dNTPs (Fermentas), 5 U/µl of Taq DNA polymerase (Roche), 0.5 pmol of
pET01S and pET01AS (Table 3), 500 ng/µl of DNA and nuclease-free distilled H₂O. The PCR reactions went through an initial extension at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, and a final extension step of 72°C for 7 min.

2.17. Splicing analysis

First, 5µl of PCR product was treated with 2 µl of ExoSAP-IT (Affymetrix). ExoSAP is made of two enzymes Exonuclease I (ExoI) to remove primers and Shrimp Alkaline Phosphatase (SAP) to dephosphorylate excess dNTPs. Reaction tubes were incubated for 15 min at 37°C and followed by enzyme inactivation for 15 min at 80°C. Then, treated products were sequenced as described in section 2.3. The sequences were aligned and analysed using Geneious 7.1.5 (Biomatters 2014) and Mesquite 2.75 (Maddison and Maddison 2011).

3. Results

To ensure that the only difference between wild type and mutant genotypes inserted in the vector pCR™4-TOPO was only the 6 bp deletion the sequences were sequenced and aligned as is showed in Figure 1.

**Figure 1.** Screening process

![Screening process](image)

*Sample one belonged to the wild type genotype while sample two showed the 6 bp deletion.*

To check the correct introduction of the fragment inside the pET01 plasmid in the transformed NEB 5α cells, digestion was performed (Figure 2).
**Figure 2.** pET01 recombinant plasmid digested with SalI and XbaI

Lane 2 and 9 pET01 vector digested with SalI and XbaI. Predicted size of insert 506 bp. Lane 1 and 7, DNA ladder 1 kb. Lane 13, negative control.

The splicing pattern was analysed in COS-7 and BE(2)-C cells. A total of 10 samples were used for RNA extraction. Then, cDNA synthesis and PCR were performed as shown in Figure 3.

**Figure 3.** PCR of the cDNA synthesis from COS and BE(2)-C cells

The results were the same for both cell lines. All samples were extended with oligo(dT) primers. Lanes 2 and 3, wild type samples. Lanes 4 and 6, samples with the 6 bp deletion. Lanes 3, and 5, negative controls for the cDNA synthesis (i.e. samples without enzymes). Lane 1, DNA Ladder 1 kb. Lane 6, negative control for PCR (i.e. without cDNA).

Finally, the spliced sequences of the samples were retrieved. Though the deletion was predicted to be part of a silencer site close to exon 17, the splicing was not altered in monkey kidney cells, Figure 4. Both wild and mutant genotypes showed the same splicing pattern where the intronic sequences (167 bp upstream and 192 bp downstream) flanking exon 17 of the APP gene were removed successfully and exon 17 remained.
a) The sequence amplified in this study was a fragment of 506 bp. This sequence contained exon 17 (highlighted in grey) and two intronic regions: 1) 167 bp upstream and 2) 192 bp downstream of the APP gene. In green, the 6 bp deletion located downstream of the exon 17.

b) The fragment retrieved from the RNA and cDNA synthesis. This fragment corresponded to the complete exon 17.

4. Discussion

The molecular causes of AD are still poorly understood. The discovery of Aβ peptide led to the formulation of the Amyloid cascade hypothesis, where mutations in APP, PSEN1 and PSEN2 have been identified as the main pathogenic variants for early onset of AD (28). Several mutations in the APP gene have been documented (Table 2). However, they only explain a very small percentage of all AD cases. This could propose that there are other major genetic players in the development of AD.

Mutations in key elements of splice sites can alter exon recognition and have strong impact on mRNA maturation and protein synthesis (22). However, the results demonstrated that the splicing process is not affected by this 6 bp deletion since both introns 16 and 17 were removed successfully and exon 17 was retained with both wild-type and mutant cell lines.

This outcome could be suggesting the following:

a) The most probable conclusion would be since the mutation is placed in an intronic region it might not be interfering in any process of splicing. So, this deletion does not appear to have any protective effect from AD. However, the patient with this mutation was diagnosed with early onset of AD and no family history of cognitive impairment has been found. So it would appropriate to identify a de novo familial mutation either PSEN1 or PSEN2 genes.
b) When the 6 bp deletion was found, Human Splicing finder (HSF), a bio-informatics tool predicted that potential branch points, enhancers motifs and potential acceptors and donators of splice sites could be altered by this mutation. However, as no change in the splicing pattern was observed it is possible that HSF mistook these possible splicing sites by pseudoexons or decoy sites. In other words, during the splicing, consensus motifs are recognized by the spliceosome. These consensus motifs are called donor (5’ss) and acceptor (3’ss) splice sites. They are usually 9 bp for the 5’ss and 14 bp for the 3’ss, where their terminal dinucleotides (GT-AG, GC-AG and AT-AC) are very conserved among genes. Given this conservation, adjacent position can vary resulting in short degenerated consensus positions. Such small motifs could be found all over the intron by chance resulting in pseudo exons (22, 29). On the other hand, HFS failed to predict correctly the interaction of the deletion and splicing. Mostly all bioinformatics tools are able to predict with high accuracy (100%) the impact of conserved splice site motifs. However, the prediction efficiency decreases (72%) when they are used to predict the impact of mutations in degenerated positions (22). This infers that even though this deletion was expected to be affecting splicing it turned out to be a false positive.

c) Even though, exon 17 is retained in both wild and mutant this deletion could still be having an effect on the ratio of isoforms produced. SR proteins could be taking longer to recognize cis-acting elements leading an alteration in the rate of the splicing process and therefore protein production. It is well-known that there are procedures that could be decreasing the level of splice variants or redirecting splicing to favor the production of other isoforms generating human diseases like in MAPT or being altering the amount of Aβ production protecting an individual to suffer from AD (30). This could be proved in further studies through RNA extraction of the patient’s brain tissue together with real time PCR.

Finally, as mentioned above there are more studies to do. Future assays must be done in the same conditions described in this project since variations in the minigene size used, various types of cells with different compositions and levels of endogenous splicing factors as well as SR protein kinases and phosphatases, and different stages of cells may contribute to the outcome of these types of studies (23).
5. Acknowledgements

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6. References


