Investigating Splicing Variants Uncovered by Next-Generation Sequencing the Alzheimer’s Disease Candidate Genes, CLU, PICALM, CR1, ABCA7, BIN1, the MS4A Locus, CD2AP, EPHA1 and CD33

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Abstract

Late onset Alzheimer’s disease (LOAD), the most common cause of late onset dementia, has a strong genetic component. To date, 21 disease-risk loci have been identified through genome wide association studies (GWAS). However, the causative functional variant(s) within these loci are yet to be discovered. This study aimed to identify potential functional splicing mutations in the nine original GWAS-risk genes: CLU, PICALM, CR1, ABCA7, BIN1, the MS4A locus, CD2AP, EPHA1 and CD33. Target enriched next generation sequencing (NGS) was used to resequence the entire genetic region for each of these GWAS-risk loci in 96 LOAD patients and in silico databases were used to annotate the variants for functionality. Predicted splicing variants were further functionally characterised using splicing prediction software and minigene splicing assays. Following in silico annotation, 21 variants were predicted to influence splicing and, upon further annotation, four of these were examined utilising the in vitro minigene assay. Two variants, rs81768 A>G in ABCA7 and a novel variant 11: 60179827 T>G in MS4A4A were shown, in these cell assays, to affect the splicing of these genes. The method employed in the paper successfully identified potential splicing variants in GWAS-risk genes. Further investigation will be needed to understand the full effect of these variants on LOAD risk. However, these results suggest a possible pipeline in order to identify putative functional variants as a result of NGS in disease-associated loci although improvements are needed within the current prediction programme in order to reduce the number of false positives.

Keywords: Late onset Alzheimer’s disease; Next generation sequencing; Splicing; Annotation; Functional variations; Minigene assays

Introduction

Late onset Alzheimer’s disease (LOAD) is an insidious neurodegenerative disease responsible for most cases of dementia in the elderly. Despite being widely studied, the disease still lacks a clear pathogenesis. However, there is a strong genetic component to LOAD with 21 disease-risk genetic regions having been identified through genome wide association studies (GWAS) [1]. Due to the nature of GWAS experimental design, the variants associated through GWAS are frequently not the causal functional variants generating the disease association. Instead, one or several variants in linkage disequilibrium (LD) with the GWAS variant are likely to be causal [2,3]. Therefore, due to the large number of the possible causative functional variants within these loci, the mechanistic role that each of the identified 21 disease-risk genes may play in the progression of LOAD remains unknown.

To identify the disease-causing functional variant(s), a number of next-generation sequencing (NGS) studies have been undertaken for the LOAD GWAS loci. Examples include CLU [4], CLU, PICALM and CR1 [5], ABCA7 [6] and ABCA7, BIN1, CD2AP, CLU, CR1, EPHA1, MS4A4A/MS4A4A6 [7]. All studies, apart from [5], have used targeted exome sequencing, variants which may be found in the noncoding and intronic regions of the loci. Exome sequencing may also miss exonic variants which are found close to exon/intron borders [8]. Therefore these studies may have overlooked mutations which could affect splicing through disrupting donor and acceptor splice sites.

Splicing plays an important role in human genetic disease. Almost a third of currently known disease-causing variants disrupt splicing, although this is likely to be an underestimate [9,10]. Aberrant splicing is implicated in many neurodegenerative disorders [11] and in LOAD, variants causing dysfunctional splicing have been found in some of the risk genes including PICALM and CD33 [12-14].
The aim of this study was to discover potentially functional causative splicing variants in the original nine genes highlighted through GWAS in 2009: CLU, PICALM, CR1, ABCA7, BIN1, the MS4A locus, CD2AP, EPHA1 and CD33. Target enrichment and next generation sequencing (NGS) were used to sequence the entire GWAS locus for each gene as identified through linkage disequilibrium. Coding and non-coding variants were prioritised for causality using functional annotation. Many of the variants were located close to intron/exon boundaries, suggesting a possible role in splicing. These variants were taken forward for further in silico investigation and experimental assessment of functionality.

Methods

NGS

Informed consent was obtained from all participants and the local Ethics Committee approved the study. 96 CERAD post-mortem confirmed LOAD brain tissue samples were obtained from the University of Nottingham Brain Bank (n=50) and the Manchester Brain Bank (n=46). This sample size gave 80% power to detect variants with a minor allele frequency (MAF) as low as 0.85% at a particular location. All samples were Caucasian, 52.8% female, with average age at onset of 70.8 years and non-APOE alleles: ε2-9.1%; ε3-57.2%; ε4-33.7%, data taken from the University of Nottingham Brain Bank. Linkage disequilibrium (LD) between the variants of interest and the GWAS variants were calculated with VCFtools [25] using 1000Genomes Phase 1 data for samples of northern European Ancestry [26].

In silico analysis of splicing

Additional in silico programs were used to assess the 21 identified splicing SNPs. Namely, ESEFinder v3 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) [27], Berkley Drosophila Genome Project Splice Site Prediction by Neural Network (BDGP) (http://www.fruitfly.org/seq_tools/splice.html) [28] and Human Splicing Finder (HSF) (http://www.uwm.edu/HSF3/) [29]. All programs were accessed March 2015.

Four potential variants were taken forward that showed differences between the two alleles in all three programs, including a score change of more than 10% between the major and the alternative allele where numerical scores were provided.

In vitro analysis of splicing

As additional brain tissue was not available for all samples in order to perform RNA extractions, as well as the previous DNA extractions already performed, the minigenes assay was selected to validate potential splicing variants. This method has been shown to have complete concordance with RT-PCR analysis of patient RNA and is a validated and reliable method for investigating splicing [30].

All methods were performed following manufacturer's protocol unless otherwise specified. The specific sample in each pool containing the heterozygous splice variant was identified by Sanger sequencing the appropriate exon(s) in each locus.

Minigene primers with the addition of SalI and XbaI restriction enzyme binding sites were designed using the relevant ABCA7, MS4A6A and EPHA1 reference sequences using Primer 3 (v.0.4.0) using the pooled data specific program, CRISP [21]. Called variants were filtered following the best practice variant detection suggested by Genome Analysis ToolKit pipeline (GATK v4.0, Broad Institute [22]. Variants were annotated using Ensembl's Variant Effect Predictor (VEP) [23] supplemented with annotations from Encyclopaedia of DNA Elements (ENCODE) project and PhastCons downloaded from the UCSC genome browser website (http://genome.ucsc.edu/ENCODE/downloads.html, accessed Nov 2012 [24]) using a custom in-house script. 21 variants identified as potentially affecting splicing (falling within 1-3 bp of exon and 1-12 bp of intron) were carried forward for further investigation. Given the difficulties identifying intronic mutations which affect branch point sites and the large number of intronic variants called, these were not taken forward in this project. Linkage disequilibrium (LD) between the variants of interest and the GWAS variants were calculated with VCFtools [25] using 1000Genomes Phase 1 data for samples of northern European Ancestry [26].

Additional Table 1: Regions of the genome (chromosome number and coordinates) to be targeted by Agilent SureSelect baits for the sequencing project.

| Gene | Transcript ID | Chromosome | Coordinates | BP |
|------|---------------|------------|-------------|----|
| CLU  | NM_001381.3  | 8          | 27 450 849-27 475 277 | 24,430 |
| PICALM | NM_007166.3 | 11 | 85 665 237-85 783 519 | 118,280 |
| CR1  | NM_000651   | 1          | 207 667 495-207 816 719 | 149,224 |
| ABCA7 | NM_019112   | 19         | 1 038 952-1 066 720 | 27,768 |
| BIN1  | NM_139343.2 | 2          | 127 778 085-127 895 723 | 117,638 |
| MS4A LD Locus | NG_016014 | 11         | 59 856 026-60 041 296 | 185,268 |
| CD2AP | NM_012120   | 6          | 47 427 281-47 601 015 | 173,734 |
| EPHA1 | NM_005232   | 7          | 143 082 382-143 110 385 | 28,003 |
| CD33  | NM_001772   | 19         | 51 718 317-51 748 546 | 30,229 |

Table 1: Regions of the genome (chromosome number and coordinates) to be targeted by Agilent SureSelect baits for the sequencing project.

Coordinates are given relative to hg19. BP refers to how many bases can potentially be covered by the design. The Ensembl transcript IDs presented were utilised in all analysis of the sequence data as well as annotation of variants.
Amplicons were cloned into pCR 2.1-TOPO vectors using the TOPO TA Cloning kit (Invitrogen, Life Technologies) before being ligated into the exon trap vector, pET01 (MoBiTech) and transformed into NEB High Efficiency 5-alpha chemically competent E. coli cells C2987L. Vector DNA was then extracted following the NucleoBond Xtra Midi Plus EF Kit in an endotoxin free environment as well as being sequenced to confirm the insert sequence was accurately cloned.

Two cell lines were obtained from the European Collection of Cell Cultures (ECCC): CV-1 in Origin, carrying SV40 (COS-7) derived from monkey African green kidney cells and human Caucasian neuroblastoma cells (BE(2)-C). COS-7 is typically used for testing minigene splicing assays as it is easy to transfect and grow, as well as being known to accurately represent the complex eukaryotic splicing environment seen in vivo. BE(2)-C cells were selected in order to investigate possible neurological-specific splicing effects due to the brain’s unique splicesome. COS-7 were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% Foetal Bovine Serum (FBS), 2 mM L-Glutamine, 100 U/ml penicillin-streptomycin and 100 U/ml fungizone and BE(2)-C were cultured in 1:1 Eagle’s Minimal Essential Medium (EMEM); Ham’s F12 with 1% non-essential amino acids, 2 mM Glutamine, 15% FBS, 100 U/ml penicillin-streptomycin and 100 U/ml fungizone. The COS-7 and BE(2)-C cells were plated out at 3.5 x 10^5 cells per plate and 6 x 10^5 cells per plate, respectively. Cells were transfected using TransFact (Promega) and incubated for 24 h. The transfection was repeated in triplicate in each cell line. Total RNA was then extracted utilising the RNeasy Mini Kit (Qiagen) with the additional column on DNAse treatment (TURBO DNA-free Kit, Ambion). Total cDNA was synthesised with AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent) using oligo(dT) primers. The cDNA of interest was then PCR amplified in a 30 μl reaction volume using LongAmp Taq (New England BioLabs) and primers specific to the pET01 vector (Forward: GATCGATTCGCTTCCTCCTG; Reverse: CACTGGAAGGTGGCCCG). The thermal cycling conditions were: 30 s at 94°C for initial denaturation, followed by 30 cycles of 15 s at 94°C for denaturation, 30 s at 59°C for annealing and 50 s at 65°C for extension and 10 m at 65°C for the final extension. Amplicons were compared by electrophoresis as well as Sanger sequenced in order to undertake a more detailed comparison.

Results

NGS study

Analyses of 245.5 million reads per pool was obtained. The eight pools of twelve passed all FastQC parameters apart from sequence duplication which is to be expected given the sequencing strategy employed.

The flagstat analysis in SAMtools showed that 99% of reads were mapped correctly and 95-99% of reads were properly paired. CRISP called 3205 variants within the nine loci, with 760 novel variants. The minor allele frequency estimates from CRISP were strongly positively correlated with frequencies found in the 1000 Genomes project indicating successful variant calling (Spearman Correlation Coefficient=0.60, p<0.0001 across all gene regions examined). Following annotation, only 126 exonic variants were called with the majority of variants being non-coding. There were 43 variants in untranslated regions (UTRs) and 44 missense variants (Supplementary Table 2). As defined above, 21 variants were predicted to affect splicing (Supplementary Table 2).

In silico analysis of splicing

All 21 variants were further annotated by three in silico programs, the results of which can be seen in (Supplementary Table 3). From this original list of 21 variants, four were predicted to be splice site variants by at least two programs by altering the donor or acceptor sites (predicted by the BDGP and HSF programs) as well as altering splicing factor binding sites (predicted by ESEfinder) (Table 2). These variants (19:1054696 and rs881768 in ABCA7, 11:60179827 in MS4A6A and rs6967171 in EPHA1) were therefore analysed in vitro in order to assess the accuracy of these predictions.

In vitro analysis of splicing

For two of the variants analysed (19:1054696 in ABCA7 and rs6967171 in EPHA1) there was no difference between the RNA extracted from cells transfected with the wild-type insert and the mutant insert in both BE(2)-C and COS-7 cell lines. This was apparent on both gel electrophoresis and sequencing of the RT-PCR products. The ABCA7 variant rs881768 A>G, however, produced different results between the wild-type and mutant constructs upon analysis of RT-PCR products, as shown in (Figure 1). The major product with both constructs (when either the A or G allele was present) had exon 32 spliced out, leaving only vector sequence. With the mutant construct there was also a minor product, where exon 32 was included, suggesting low levels of exon 32 inclusion with the minor allele haplotype. This was evident in both COS-7 and BE(2)-C cell lines.

The MS4A6A variant at genomic position 11: 60179827 T>G also showed some differences between constructs, presented in (Figure 2). The major product with the wild-type construct, as predicted, contained both the vector exons and exon 4 of the gene. There was also a minor product present which contained just the vector sequence, suggesting low levels of a gene product with this exon spliced out. The only product with the mutant construct showed exon 4 spliced out. These results were obtained with both COS-7 and BE(2)-C cell lines.

Discussion

A 2013 GWAS meta-analysis for LOAD used over 74000 samples to identify 11 new disease-risk genes and confirmed 10 previous susceptibility loci (CLU, PICALM, CR1, ABCA7, BIN1, the MS4A Locus, CD2AP, EPHA1, and CD33). In vivo association studies of the variants’ impact on splicing, four variants were put forward for in vitro confirmation using hybrid minigenes in the pET01 vector. Only two of the variants were confirmed to be functional; rs881768 A>G in ABCA7 and novel variant 11: 60179827 T>G in MS4A6A, part of the
MS4A locus. This suggests that the methods currently used to prioritize potential functional variants need further refinement.

The synonymous variant rs881768 is located at the first base of exon 32 in ABCA7 (transcript ID NM_019112) and is in linkage disequilibrium with the ABCA7 GWAS variant (D’=0.824, Table 2). In silico predictions suggest that the G allele creates a new donor site with a similar score to the original donor site, located only 126 bp away (a score of 0.98 compared with 0.99) and changes the binding of two exon splicing enhancer (ESE) proteins (Table 2). These predictions suggest the G allele could activate a cryptic splice site or cause a change in protein isoform ratios. However, population data from 1000 Genomes

### Table 2: Prioritised splicing variants from the NGS study selected for further experimental investigation.

| Gene     | Genomic Location (rsID) | Transcript location | Ref/Alt | MAF    | D’   | ESEfinder | BDGP                  | Human Splicing Finder                                                                 | Combined predicted consequence                                                                 |
|----------|-------------------------|---------------------|---------|--------|------|-----------|-----------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| **ABCA7** (NM_019112) | 19:1054696 (-) | Intron 28-29 +3 bp | G/C     | 0.010  | NA   | SRSF2 site gained | Known functional donor site with a score of 0.91 reduced to a score of 0.38 | Alteration of an intrinsic ESS site.                                                                 | Change in ESE and intrinsic ESS site could change transcript isoform ratio.                   |
|          | 19:1056066 (rs881768) | Exon 32 1 bp        | A/G     | 0.250  | -0.824 | SRSF1 site gained, SRSF6 site lost | Novel donor site created with a score of 0.98 Strengthens known functional acceptor site score from 0.56 to 0.76 | A cryptic donor site is activated.                                                                 | Activation of donor site at start of exon might result in exon skipping.                   |
| **MS4A6A** (NM_152852) | 11: 60179827 (-) | Intron 4-5 +4 bp    | T/G     | 0.480  | NA   | SRSF5 sites lost | Known functional donor site with a score of 0.66 lost | Activation of intronic cryptic acceptor site, and intronic cryptic donor site and creation of intrinsic ESE site. | Loss of existing donor site could result in exon skipping. However, activating intronic acceptor or donor site could result in a change in the sequence included in the exon. |
| **EPHA1** (NM_005232)  | 7:143391774 (rs6967117) | Exon 17 +1bp        | T/C     | 0.060  | 0.75 | SRSF1 site gained | Novel donor site created with score of 0.49. | A cryptic donor site is activated.                                                                 | Activation of donor site at start of exon might result in exon skipping.                   |

Figure 1: (A) RT-PCR products generated using total RNA extracted from BE(2)-C cells containing both the wild-type (wt) and mutant (mut) constructs for ABCA7 variant rs881768. (B) Sequence of product generated by the major and minor allele (wt and mut) containing only vector exons. (C) Sequence of mutant construct (mut) minor allele product containing exon 32 suggesting low levels of exon 32 inclusion in the presence of the minor allele haplotype.
shows that the G allele is actually the ancestral allele with a European population allele frequency of 0.44. Additionally, score predictions for the acceptor site shows that the A allele has a lower score than the G allele (0.56 compared to 0.76) which could lead to exon skipping. This makes in silico functional prediction of this variant very difficult.

In the minigene assays the G allele causes low levels of exon 32 to be incorporated into the transcript, while the A allele causes exon 32 to be spliced out. Expressed sequence tag (EST) databases such as GTEx portal [31] show exon 32 is incorporated in most transcripts and utilising this transcript data to examine the effect of rs881768 shows that this variant has no effect on splicing in this database. ABCA7 has an interesting pattern of alternative splicing, with many introns consisting of multiples of three, potentially allowing in-frame addition and deletion of introns, although no such transcripts have been identified. Exon 32, however, does results in a frame-shift change creating a termination codon in the next exon and causing early truncation of the protein. This truncated protein is non-functional as it lacks the last five transmembrane domains and the second nucleotide binding domain and may be targeted for nonsense mediated decay. This would prevent any truncated proteins being expressed causing sole expression of the complete isoform, explaining the lack of this truncated protein in databases such as GTEx.

The novel variant identified in MS4A6A, part of the MS4A locus, 11:60179827 T>G is found 4 bp into the intron between exon 4 and 5. The G allele removes the native donor site for exon 4/intron 4 possibly causing exon skipping (Table 2). However, a cryptic donor site is activated and the binding of two ESE proteins are affected, potentially changing the sequence included in the transcript. The minigene assays show that the G allele does appear to cause exon skipping, creating transcripts without exon 4. However, the T allele, while mostly producing transcripts which contain exon 4, also produces a small proportion of transcripts without exon 4. Transcripts without exon 4 appear to be produced by both alleles despite the removal of exon 4 generating a truncated protein which lacks two transmembrane domains and one noncytoplasmic domain. Although the G allele does cause exon 4 to be excluded through obliterating the donor site, there must be additional alternative splicing regulation mechanisms occurring within the in vitro assay, as well as in vivo, which affect the levels of each isoform being produced explaining the presence of both isoforms in the presence of the T allele.

To fully explore the role both of these variants play in splicing, direct analysis of RNA samples from affected tissues from LOAD individuals with the variants should be examined. Unfortunately for rare variants in LOAD this tissue is not always readily available. Epstein-Barr transformed cell lines used for 1000 Genomes project, however, are a possible alternative. Targeted sequencing of RNA extracted from these cell lines selected to be homozygous for different alleles could be compared to the results of the minigene assays. This would determine the role of these splicing variants in an environment where the whole genome is present, rather than just the fragment inserted in the minigene assay. There is also publicly available RNA-seq data from these cell lines (see the GEUVADIS project [32]); unfortunately coverage for many of the LOAD risk loci is poor.
controls [33,34]. Additionally, small nuclear and heterogeneous nuclear ribonucleoproteins have been shown to be disrupted in LOAD [35,36]. Therefore the role of splicing in LOAD disease pathology should not be ignored.

Identifying variants that have a functional role in complex diseases is a challenge [37]. This is particularly true for next generation sequencing studies which identify large numbers of potential functional variants. The problem arises while attempting to prioritise the variants for further functional assays or experiments. Current in silico databases have minimal experimental information available. The annotation programs used need to be chosen carefully in order to correctly assign potential functionality and ensure pathogenic variants are found.

Prediction algorithms for exonic and intronic splicing enhancer and silencer sites are less robust than programs predicting disruptions of 3' and 5' consensus splice site regions. This is largely because more is known about the 3' acceptor and 5' donor consensus sequences, thus allowing better prediction models to be built [38]. Due to this, the initial selection of splicing variants in this study selected variants within 1-3 bp of an exon or 1-12 bp of an intron, biasing the selection towards variants that may disrupt consensus splice sites. The BDGP and HSF prediction programs used in this study both examine the effects of mutations on 3' (acceptor) and 5' (donor) consensus sequences. The two variants that had an effect on splicing in vitro were both predicted to affect acceptor and or donor consensus sites by BDGP and HSF (Table 2).

The recognition of exon and intron borders in pre-mRNA by the spliceosome does not simply involve identifying the correct consensus 3' and 5' splicing sequences. A multitude of splicing regulatory proteins and ribonucleoproteins interact to precisely control splicing and influence the levels of different transcript isoforms created [39]. Many variants that disrupt splicing are found deep within exons and introns and affect splicing through the disruption of exonic or intronic splicing enhancers or silencers [40]. Through limiting the analysis to variants located near consensus splice sites, as in this study, these mutations will be missed. With the advent of additional functional experimental studies, predictions of mutations influencing the binding of RNA proteins can only improve and would be useful for future work. This issue has recently been addressed by ANNOVAR, with the option to include bulk annotations from the SPIDEX dataset. This dataset provides an improved algorithm for detecting potential splicing variants [41]. However, a review of the method suggests that two other prediction methods for exonic splicing regulatory elements may perform better [42].

Conclusion

Pooled NGS with target enrichment successfully identified potential functional splicing variants in nine gene regions associated with LOAD. Through targeting the entire genomic sequence, we were able to investigate variants in these regions which potentially affect consensus splice sites in silico and in vitro. Improvements are needed in current splicing prediction programs to reduce the number of false positives which are taken forward for analysis as well as reducing the number of false negatives discarded prior to further investigation. Further work is needed to fully clarify the role that the variants rs881768 (ABCA7) and 11:60179827 T>G (MS4A4E) may have in LOAD in vivo.

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