Genetic variation affecting exon skipping contributes to brain structural atrophy in Alzheimer's disease

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Abstract

Genetic variation in cis-regulatory elements related to splicing machinery and splicing regulatory elements (SREs) results in exon skipping and undesired protein products. We developed a splicing decision model to identify actionable loci among common SNPs for gene regulation. The splicing decision model identified SNPs affecting exon skipping by analyzing sequence-driven alternative splicing (AS) models and by scanning the genome for the regions with putative SRE motifs. We used non-Hispanic Caucasians with neuroimaging, and fluid biomarkers for Alzheimer's disease (AD) and identified 17,088 common exonic SNPs affecting exon skipping. GWAS identified one SNP (rs1140317) in HLA-DQB1 as significantly associated with entorhinal cortical thickness, AD neuroimaging biomarker, after controlling for multiple testing. Further analysis revealed that rs1140317 was significantly associated with brain amyloid-β deposition (PET and CSF). HLA-DQB1 is an essential immune gene and may regulate AS, thereby contributing to AD pathology. SRE may hold potential as novel therapeutic targets for AD.

Introduction

Genome-wide association studies (GWAS) have made a significant contribution to our knowledge of genetic variants linked to human complex diseases by discovering several thousand frequently-occurring susceptibility loci (1, 2). The risk loci predicted by GWAS represent weak effect (per-allele odds ratio < 2) and require further functional analysis to identify actionable loci (3, 4). The strength of GWAS is the ability to analyze the entire genome agnostically for common alleles associated with a disease, but its weaknesses are the lack of a priori biological hypothesis to guide inquiry from association to underlying functional variants and inability to take into account non-genetic biological variation. These points can be addressed by focusing on alternative splicing (AS) as a biological mechanism regulating gene expression and influencing phenotypic variation (5-7). AS is regulated in part by DNA sequence motifs, called splicing regulatory elements (SREs) (8, 9). When a single variation (i.e., SNP (single nucleotide polymorphism)) occurs at any position of a splicing regulatory element (SRE), it can change the accuracy of spliceosome in recognizing splice sites, possibly generating harmful protein product (10-12).

SREs, six base-pairs in length, are known to influence the probability of an AS event. SREs occur in both exons and introns. They can either enhance the frequency of splicing at a nearby splice site or inhibit it. There are thus four types of SREs depending on their function and where they occur: exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs). As DNA sequence elements, it would be valuable to identify specific genetic variants (i.e., SNPs) in SREs that influence splicing outcomes, potentially leading to different ratios of mRNA isoforms or exon skipping between phenotype cases or tissues.

Genetic variation occurring in cis-regulatory elements (i.e., splice sites, SREs, and branch points) results in exon skipping and undesired protein products, and thus forms the genetic basis of 15-50% of heritable human diseases (12-14). Furthermore, alternative splicing is a key contributor to increase a biological complexity by producing multiple mRNAs from one gene. Some of mRNAs are tissue-specific, especially critical for brain function, and genes associated with disease pathogenesis are alternatively spliced (15). For example, missing exon 5 of *PSEN2* were observed in in both early and late onset AD patients. *Tau* isoforms including exon 10 (4R-*tau*) are known to lead to tau aggregation (16).

APOE4 also generates multiple mRNA isoforms that are differentially expressed in normal versus AD patient tissues (17).

Alzheimer's disease (AD) is an irreversible, progressive brain disorder characterized by the formation of amyloid plaques and neurofibrillary tangles in the brain. With the heritability of AD estimated to be as high as 80%, genetic variation may play a major role in AD pathogenesis (18-20). However, despite the success of large-scale GWAS, where more than 22 susceptibility genes for AD including the best established and the most significant genetic risk factor (*APOE* ε4) were discovered and validated, a substantial proportion of the heritability remains to be unexplained for AD (21). Additionally, variation affecting AS is understudied in AD. In this study, we developed a splicing decision model to identify actionable loci affecting exon skipping among common SNPs by analyzing sequence-driven AS models and by scanning the genome for the regions with putative SRE motifs. Then, using non-Hispanic Caucasians with HRC-based imputed GWAS and AD-related neuroimaging (MRI and PET scans) and fluid (CSF) biomarkers from the Alzheimer's Disease Neuroimaging Initiative (ADNI), we examined exonic SNPs in SREs as a mechanism to understand how genetic variants contribute to AD (22).

Methods

Identification of SNPs in splicing regulatory elements associated with exon skipping events

We have built a computational pipeline for genome-wide identification of SNPs occurring within SRE sites that are related to exon skipping (23, 24). Figure 1 summarizes the proposed method (defined as a "splicing decision model") for identifying SNPs affecting SREs with exon skipping. We identified exon skipping events by using alignment information (25, 26) for four AS datasets from the UCSC genome browser (27, 28): mRNAs from GenBank (29), Ensembl Gene Predictions (30), AceView Gene Models (31), and UCSC known genes (32). Using a set of predicted hexameric SRE motifs, including ISEs, ESEs, and ESSs obtained from the publication (33), we searched for all potential SRE sites that are perfectly matched with any of these hexamers in intragenic regions. We combined genotype data, SRE regions and skipping of the adjacent exon, and identified intragenic SNPs in SREs that potentially affect exon skipping.

Alzheimer's Disease Neuroimaging Initiative (ADNI)

All individuals used in this study were participants from the ADNI. As detailed previously, inclusion and exclusion criteria, clinical and neuroimaging protocols, and other information about ADNI can be found at www.adni-info.org (22). Demographic information, raw neuroimaging scan data, *APOE* and whole-genome genotyping data, neuropsychological test scores, and diagnostic information are publicly available from the ADNI data repository (http://www.loni.usc.edu/ADNI/).

Neuroimaging analysis

For T1-weighted brain MRI scans, two widely employed automated MRI analysis techniques were independently used to process MRI scans: whole-brain voxel-based morphometry (VBM) and FreeSurfer software, as previously described (34). [^{18}F] Florbetapir scans were pre-processed as described (35). [^{18}F] Florbetapir PET scans were intensity normalized by the whole cerebellum. These normalizations yielded standardized uptake value ratio (SUVR) images.

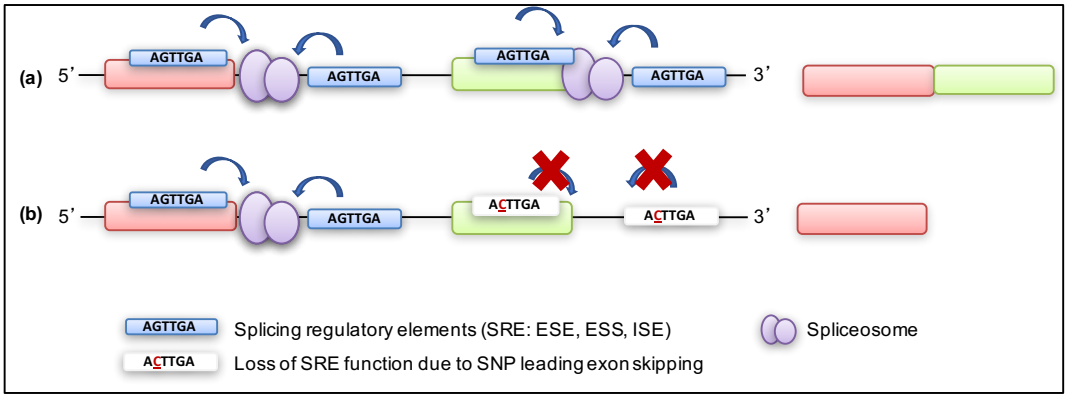


Figure 1. Diagram of computational methods for identifying SNPs in SREs with exon skipping events. (**a**) Normal splicing of two exons (red and green) is processed by SREs, leading to the inclusion of two exons in mature mRNA. (**b**)

Abnormal splicing of the green exon is led by SREs with the variant at the second position of SRE (white hexamer) leading to its skipping from mature mRNA.

Genotyping and imputation

Genotyping for ADNI was performed using the Illumina Human610-Quad BeadChip, Illumina HumanOmni Express BeadChip, and Illumina HumanOmni 2.5M BeadChip platforms. *APOE* genotyping was separately obtained using standard methods to yield the *APOE* ε4 allele defining SNPs (rs429358, rs7412) (36). As the ADNI used different genotyping platforms, we imputed un-genotyped SNPs separately in each platform using MACH and the HRC (Haplotype Reference Consortium) data as a reference panel. Before the imputation, we performed standard sample and SNP quality control procedures as described previously (37): (1) for SNP, SNP call rate < 95%, Hardy-Weinberg test $p<1 \times 10^{-6}$, and minor allele frequency (MAF) < 1%; (2) for sample, sample gender and identity check, and sample call rate < 95%. Furthermore, to prevent spurious association due to population stratification, we selected only non-Hispanic Caucasian participants using HapMap data and multidimensional scaling (MDS) analysis (www.hapmap.org) (38). Imputation and quality control procedures were performed as described previously (39). After the imputation, we imposed an r^2 value equal to 0.30 as the threshold to accept the imputed genotypes.

Imaging genetics analysis

We performed genome-wide association analysis (GWAS) using HRC-based imputed genotype data and AD-related imaging biomarker, mean entorhinal cortical thickness, as an endophenotype. In order to investigate further the association of candidate SNPs with quantitative imaging phenotypes, we performed a whole-brain surface-based analysis using the SurfStat software package (http://www.math.mcgill.ca/keith/surfstat/) and multivariate models for cortical thickness on the brain surface on vertex-by-vertex bases. We used age at baseline, sex, years of education, MRI field strength, and total intracranial volume (ICV) as covariates. Correction for multiple comparisons was performed using the random field theory (RFT) correction method at a 0.05 level of significance.

Results

Using the splicing decision model, we identified 17,088 exonic SNPs affecting exon skipping (MAF >1%) from HRC-based imputed ADNI GWAS data. Table 1 shows the demographic information for 1,559 non-Hispanic Caucasian ADNI participants (362 cognitively normal older adults (CN), 95 significant memory concern (SMC), 281 early mild cognitive impairment (EMCI), 511 late MCI (LMCI), and 310 AD) with GWAS data and MRI scans at baseline.

Table 1. Demographic information for 1,559 ADNI participants

	CN	SMC	E-MCI	L-MCI	AD
N	362	95	281	511	310
Age mean	74.7	71.8	71.1	73.5	74.7
(SD)	(5.5)	(5.6)	(7.3)	(7.6)	(7.8)
Sex (M/F)	192/170	39/56	156/125	318/193	176/134
Education mean	16.3	16.8	16.1	16.0	15.2
(SD)	(2.7)	(2.6)	(2.7)	(2.9)	(3.0)
APOE ε4-/ε4+	264/97	63/32	161/119	230/291	104/206

We performed GWAS for the exonic SNPs in SREs affecting exon skipping to identify genetic variation contributing to brain structural atrophy, mean entorhinal cortical thickness, in AD. Figure 2 displays quantile-quantile and Manhattan plots.

The genomic inflation factor is 1.045, suggesting no evidence of systematic inflation of *p*-values (**Fig. 2 (a)**). We identified one SNP as significantly associated with entorhinal cortical thickness after controlling for multiple testing using Bonferroni correction (corrected $p<0.05$; red horizontal line) (**Fig. 2 (b)**). The missense SNP (rs1140317) is within *HLA-DQB1*. As shown in **Figure 3**, the SNP (rs1140317) exists within two different exonic splicing enhancers (ESEs) (CCA**C**CT and A**C**CTCG) located in the second exon marked with the rectangle (as reversely transcribed), which is skipped in mRNA transcript (ENST00000399082). Furthermore, we note that the putative function of the skipped exon is a coding region translated into the MHC_II_beta domain (PF00969).

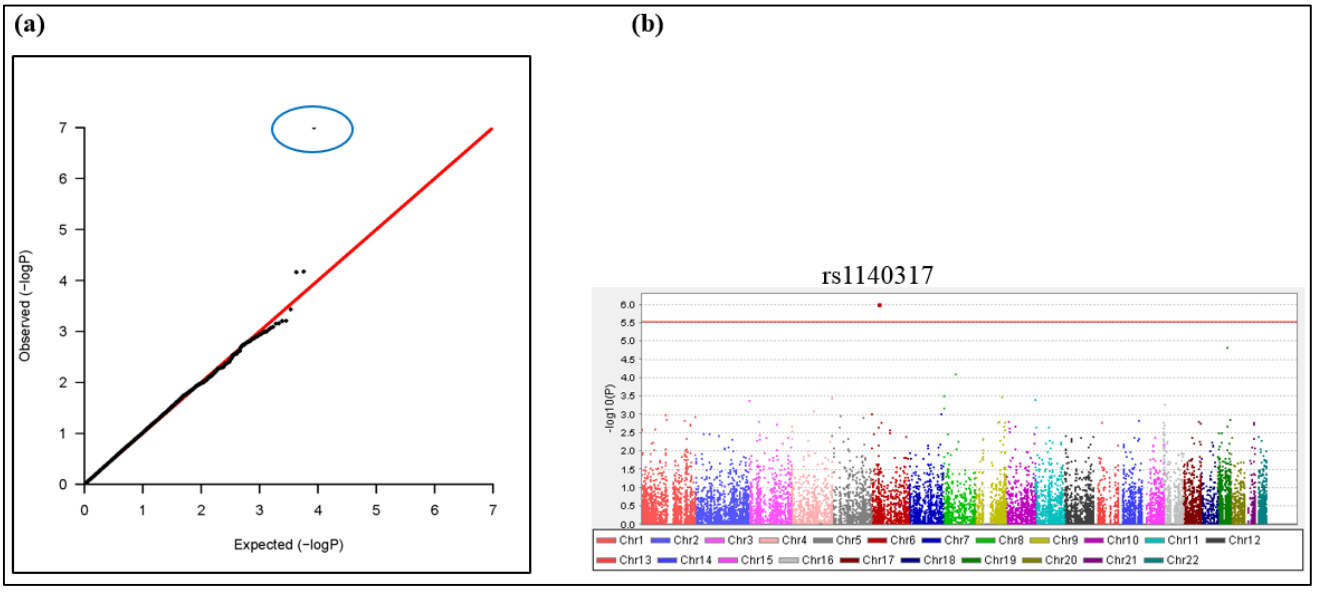
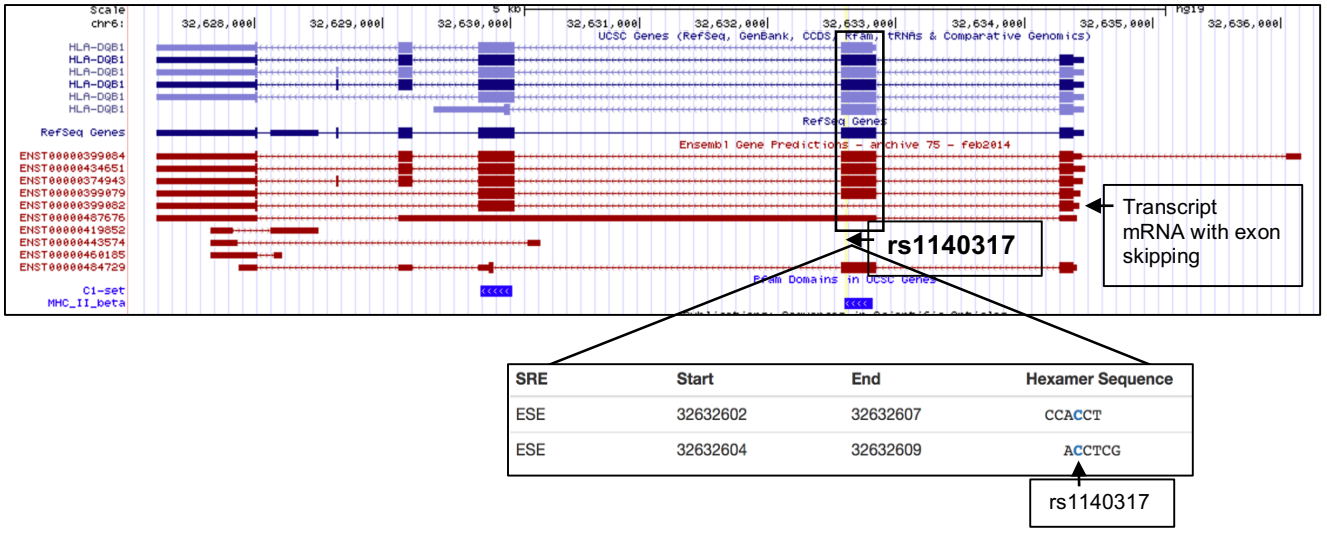


Figure 2. Quantile-quantile (a) and Manhattan (b) plots for entorhinal cortical thickness



SRE	Start	End	Hexamer Sequence
ESE	32632602	32632607	CCACCT
ESE	32632604	32632609	ACCTCG

rs1140317

Figure 3. Model of exon skipping affected by rs1140317 SNP in two exonic splicing enhancers and potential impact of the skipped exon on the MHC_II_beta domain region (PF00969) of *HLA-DQB1*.

As a novel association, the entorhinal cortical thickness increases with the number of minor alleles (A) of rs1140317 (p=1.04 x 10^{-7}) (**Fig. 4**), which is the allele (A) disrupting SRE function, leading to the exon skipping.

The novel genome-wide significant SNP (rs1140317) was analyzed further to examine possible associations in AD. Figure 5 displays the results of the main effect of rs1140317 using surface-based analysis of baseline MRI scans. The unbiased, detailed whole-brain analysis of rs1140317 using multivariate regression models identified significant clusters in the bilateral temporal including entorhinal cortex, parietal, and frontal lobes (corrected p<0.05), where individuals carrying minor alleles showed greater mean cortical thickness compared with the participants carrying no minor allele (**Fig. 5**). No significant cortical regions were observed at the same statistical threshold in the negative contrast.

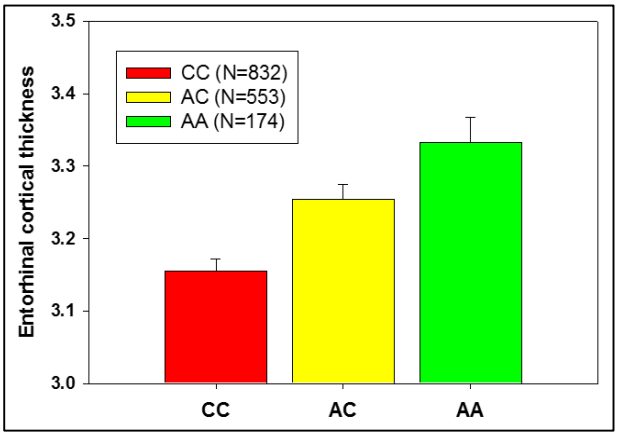


Figure 4. Association of rs1140317 in *HLA-DQB1* with entorhinal cortical thickness (p=9.75 x 10^{-7})

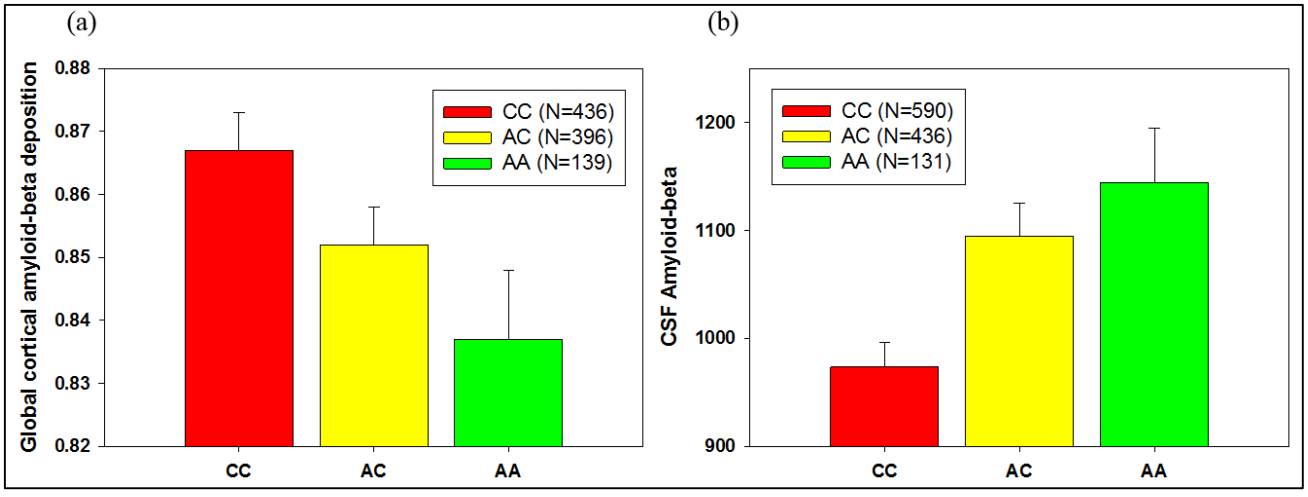


Figure 5. Association of rs1140317 in *HLA-DQB1* with cortical atrophy on the brain surface measured by MRI scans from whole brain surface-based analysis

In order to investigate further the effect of rs1140317 on other AD-related imaging and fluid biomarkers, we performed an association analysis of rs1140317 with amyloid-beta (Aβ) measures, AD hallmark, in [^{18}F] Florbetapir PET and cerebrospinal fluid (CSF). The analysis revealed that rs1140317 was also significantly associated with global brain cortical amyloid-β deposition (**Fig. 6 (a)**; p=4.60 x 10^{-3}) and CSF $A\beta_{1-42}$ levels (**Fig. 6 (b)**; p=3.69 x 10^{-3}), suggesting that the minor allele of rs1149317 confers protection.

Discussion and Conclusion

Eukaryote cells utilize alternative splicing mechanism to generate various transcript isoforms by differently assembling exons in mature mRNAs without introns. These mature mRNAs are often tissue-specifically and disease specially produced from one gene. As the splicing process in assembling exons are generally regulated by cis-regulatory elements including splice sites and splicing regulatory elements, the genetic variation (i.e., SNP) occurred within these elements leads to the undesired protein products in many complex diseases including cancers (40). Recently, we have developed methods for annotating SNPs within splicing regulatory element regions associated with exon skipping events and investigated its utility for identifying disease-associated loci (23, 24). In principle, our splicing decision methods are similar to Splicing Quantitative Trait Loci (sQTL) in terms of using a regression model to identify responsible SNPs to splicing alteration. However, the strength of our approach is to utilize SRE as a mechanism that can explain how SNPs affect the splicing. Our methods not only propose a molecular mechanism-driven annotation for SNPs but also a new way for identifying functional roles of coding and intronic SNPs using SRE. Intronic SNPs within splicing regulatory

elements were enriched among established SNPs in human complex traits. Furthermore, certain SNPs in splicing regulatory elements were extremely differentiated among populations. These studies emphasize an importance of alternative splicing in many human complex traits and diseases, and its usefulness for identifying disease-associated variation in the human genome. We note that the protein products of the changed splicing can characterize the genetic basis of inter-individual variability providing mechanistic clues to the pathophysiology of human diseases and traits.

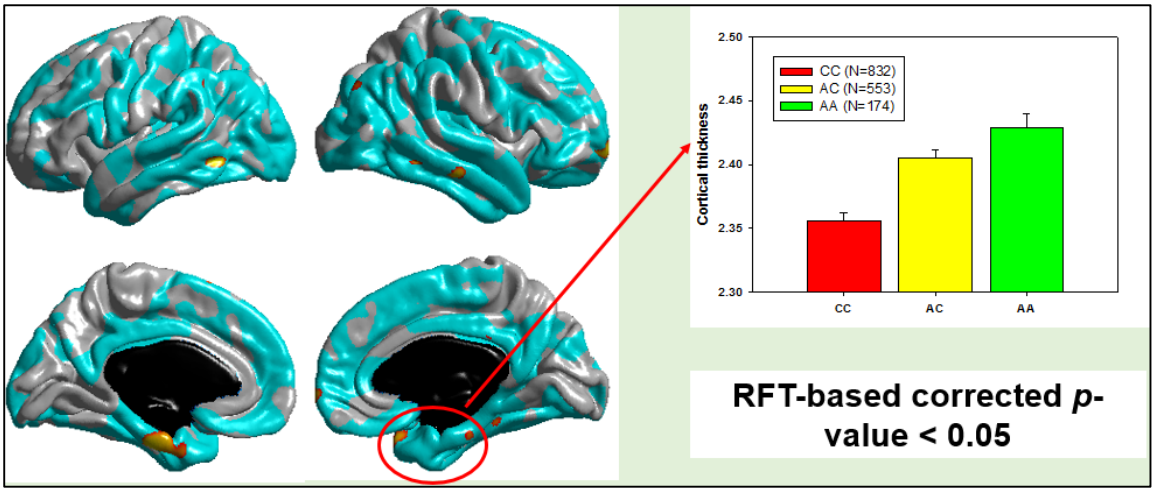


Figure 6. Association of rs1140317 in *HLA-DQB1* with amyloid-β measurements from [^{18}F] Florbetapir PET and cerebrospinal fluid biomarkers (CSF): (a) global cortical amyloid-β deposition (p=4.60 x 10-3) and (b) CSF $A\beta_{1\text{-}42}$ levels (p=3.69 x 10^{-3})

In this study, we investigated the utility of the splicing mechanism for one complex brain disease, Alzheimer's disease. GWAS identified one SNP (rs1140317) in *HLA-DQB1* as significantly associated with mean entorhinal cortical thickness in Alzheimer's disease, which starts in the entorhinal cortex. Further analysis revealed that rs1140317 was significantly associated with amyloid-β deposition, which is involved in Alzheimer's disease as a neuropathological hallmark found in the brains of Alzheimer patients. The *HLA-DQB1* (major histocompatibility complex, class II, DQ beta 1) gene provides instructions for making a protein that plays a central role in the immune system. Known AD susceptibility genes (increasing or decreasing the risk for AD) have been involved in immunity and inflammation.

Although more genes are alternatively spliced in the brain and several exons of AD-related susceptibility genes such as APP and APOE are brain-specially skipped, it is necessary to do a follow-up study in order to validate our findings. In the future, using several AD studies such as the Religious Order Study and the Rush Memory and Aging Project study (ROS/MAP), which have collected brain-tissue specific RNA-Seq, GWAS, and MRI data, we will validate our findings by performing an integrative analysis to identify exon skipping events using RNA-Seq, investigate the effect of identified SNPs on exon skipping events, and replicate our findings using the independent data set.

In conclusion, our splicing decision model enabled to identify significant associations of AD-related neuroimaging and fluid biomarkers with one missense exonic SNP in *HLA-DQB1*, an essential immune gene, that may regulate alternative splicing and thereby contribute to AD pathology. SRE may hold potential as novel therapeutic targets for AD. Our results warrant further investigation in a larger independent cohort.

Acknowledgments

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