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Alzheimer's disease genetics and *ABCA7* splicing

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Abstract

Both common and rare polymorphisms within *ABCA7* have been associated with Alzheimer's disease (AD). In particular, the rare AD associated polymorphism rs200538373 was associated with altered *ABCA7* exon 41 splicing and an AD risk odds ratio of ~1.9. To probe the role of this polymorphism in *ABCA7* splicing, we used minigene studies and qPCR of human brain RNA. We report aberrant *ABCA7* exon 41 splicing in the brain of a carrier of the rs200538373 minor C allele. Moreover, minigene studies show that rs200538373 acts as a robust functional variant *in vitro*. Lastly, although the *ABCA7* isoform with an extended exon 41 is predicted to undergo nonsense mediated RNA decay, this was not supported by qPCR analyses, which showed relatively normal ABCA7 mRNA levels in the carrier of the rs200538373 minor C allele. In summary, rs200538373 is a functional polymorphism that alters *ABCA7* exon 41 splicing without grossly altering the level of ABCA7 mRNA.

Keywords

Alzheimer's; ABCA7; Genetics; SNP; Splicing

Introduction

AD currently affects as many as 5.1 million people in the United States [1]. As 'baby boomers' age, AD prevalence is projected to triple by the year 2050 unless a treatment is found [1]. Since pharmacologic agents based on genetic mechanisms are more likely to successfully transition to drugs approved by the Food and Drug Administration [2], we seek to elucidate the actions underlying AD-associated SNPs.

Single nucleotide polymorphisms (SNP)s in *ABCA7* were identified as AD risk factors in several studies [3–6]. These SNPs included several common SNPs that were associated with modest AD risk, including rs3764650, rs4147914, rs3752246, and rs4147929 [3–5]. These SNPs were also found to associate with *ABCA7* expression, cortical and hippocampal atrophy, as well as β-secretase activity in cells expressing the amyloid-β protein precursor (AβPP)-Swedish mutation [3, 7–9]. In addition to these common ABCA7 SNPs, several rare

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Conflict of Interest Statement

The authors have no conflict of interest to report.

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ABCA7 SNPs, including rs200538373, were associated with AD at odds ratios as high as 1.9 [6].

The role of ABCA7 function itself in AD is unclear. Recent findings using human brain show *ABCA7* is expressed at low levels in several cell types, including neurons and microglia [10]. ABCA7 has been implicated in lipid transport, phagocytosis and Aβ homeostasis [11–17].

Here, we sought to better understand the role of the rare SNP, rs200538373, which was associated with *ABCA7* exon 41 splicing in blood [6]. We extend these prior findings by reporting an aberrant 14 bp extension of exon 41 in the brain of an individual that was heterozygous for this SNP. The hypothesis that this SNP is functional was supported by *in silico* modeling and by an *ABCA7* minigene study, which demonstrated that rs200538373 acts to alter exon 41 splicing. Lastly, *ABCA7* expression in a carrier of the minor allele of rs200538373 was similar to that of other brain samples; this finding is inconsistent with hypothesized nonsense mediated RNA decay for this isoform, suggesting that the likely action for this SNP is altered splicing leading to a truncated ABCA7 protein.

Materials and Methods

Ethics statement

This work was conducted under the approval of the University of Kentucky Institutional Review Board.

Human brain tissue

RNA was purified from human anterior cingulate brain samples (supplied by the University of Kentucky AD Center Neuropathology Core) and converted to cDNA as previously described [18–20]. The AD status of the brain donors was determined by the AD Center Neuropathology and Clinical Cores by using guidelines that included evaluation of neurofibrillary tangles and neuritic senile plaques as well as cognitive status [21–23]. Age at death for the cognitively intact, i.e. non-AD donors, was 82.6 ± 1.6 (mean ± SE, n = 28) while AD donors were 81.7 ± 1.2 (mean ± SE, n = 28). The post-mortem interval (PMI) for non-AD and AD donors was 2.7 ± 0.2 (n = 28) hours and 3.4 ± 0.1 (n = 28), respectively.

Genotyping

DNA samples were genotyped for the indicated polymorphisms by using FAM and VIC dye-labeled probes (Invitrogen, Carlsbad, CA) and TaqMan polymerase chain reaction (PCR) (Bio-Rad, Hercules, CA).

Splicing assay

ABCA7 exon 41 splicing was assessed by PCR coupled to polyacrylamide gel electrophoresis (PAGE). Reactions contained a sense primer corresponding to sequence within exon 40, i.e., 5′-CCGTGGGCAGAGGATG-3′ and an antisense primer corresponding to exon 42 sequence, i.e., 5′-TCGGATTGAGGGCAGTATC-3′. Each 20 μL reaction mixture contained ~20 ng of cDNA, 25 pmole of each primer, and Platinum Taq

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(ThermoFisher) and was subjected to a PCR profile of 30 cycles at 95°C for 15 s, 59°C for 30 s, and 72°C for 20 s. PCR products were separated on 10% polyacrylamide gels and detected by SYBR gold fluorescence (ThermoFisher) as per manufacturer's protocol. Each sample was analyzed twice and reactions lacking cDNA template were analyzed in parallel to check for PCR product contamination.

Maximum entropy scores

The sequences of the *ABCA7* exon 41 isoforms were scored for 5′ splice site favorability by using an online prediction tool ((http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html [24]. Briefly, this algorithm was trained on large datasets of human splice sites to calculate a log odds ratio for a splicing score for input sequence [24]. A higher score correlates with a more favorable splice site [24, 25].

RNA splicing assay

ABCA7 minigenes for each rs200538373 allele were generated by PCR and contained exon 41, intron 41, and exon 42 in their entirety, cloned into pcDNA3.1 (ThermoFisher). Minigene construction used a sense primer corresponding to sequence at the start of exon 41, i.e., 5′-TGTTTTGGGCTGCTGG-3′ and an antisense primer corresponding to sequence at the end of exon 42, i.e., 5′-CTGGGCAACCTGGGC-3′. Sequencing confirmed inserts were accurate and complete for each allele and differed only for rs200538373 alleles. Human Be(2)-M17 neuroblastoma cells (ATCC, Manassas, VA) were maintained in Opti-MEM I reduced-serum medium supplemented to 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin with humidified 5% carbon dioxide at 37°C. For transfections, cells (1×10^6) were plated in 350 μL media in a 24-well plate, allowed to grow 24 hrs and then transfected in triplicate by using 2 μg of allele-specific *ABCA7* minigene vector and Lipofectamine 3000, as per manufacturer's protocol (ThermoFisher). Forty-eight hours after transfection, RNA was prepared and reverse transcribed using random hexamers and Superscript III as described previously [26]. *ABCA7* expression from each minigene was detected by PCR using a pcDNA3.1 vector-derived forward primer (5′-ACTAGTCCAGTGTGGTGGAATTGCC-3′) and exon 42-derived reverse primer (5′-TCGGATTGAGGGCAGTATC-3′).

Quantitative PCR assay

The quantification of *ABCA7, synaptophysin, ITGAM* and *AIF1* expression in these samples has been described previously [7, 26, 27]. Briefly, 20 μL reactions contained 1μM of each primer, 1x PerfeCTa SYBR Green Super Mix (Quanta Biosciences), and approximately 20 ng cDNA generated from anterior cingulate RNA. PCR was conducted using an initial 2-minute incubation at 95°, followed by cycles of 10 seconds at 95°, 20 seconds at 60°, and 20 seconds at 72°. Experimental samples were amplified in parallel with serially diluted standards that were generated by PCR of cDNA followed by purification and quantitation by UV absorbance. Results from samples were compared relative to the standard curve to calculate copy number in each sample. Real time assays were performed twice and the average copy number was used for further analyses.

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Statistical Analysis

ABCA7 expression was analyzed by linear regression (SPSS, version 23). Each model included AD status and rs3764650 genotype. In addition, since *ABCA7* is expressed in both microglia and neurons [16, 17], we included expression of either (i) a neuronal gene (*synaptophysin*), (ii) microglial genes (geometric mean of *ITGAM* and *AIF1),* or (iii) both the neuronal and microglial genes (SPSS, v. 23). The geometric mean of *ITGAM* and *AIF1* was used, as opposed to the arithmetic mean, because *AIF1* levels were markedly higher than *ITGAM* levels. To generate a normal distribution of the data, the square root of the copy number data was used for the linear regression analyses; the square root approach was validated by the Shapiro-Wilk test for normality. We also analyzed the expression results for variation in sample mRNA content by dividing mRNA copy number by the geometric mean of two constitutively expressed genes, i.e., *RPL32* and *EIF4H* [26]. To generate a normal distribution as assessed by Shapiro-Wilk, regression analyses used the square root of these values. Since our cohort of samples had only one rs3764650 minor allele homozygous individual, we used a dominant model for this analysis, i.e., rs3764650 minor allele carriers were considered as a single group.

Results

Rs200538373 is a rare intronic *ABCA7* variant that was associated with AD risk and with exon 41 splicing in blood [6]. To confirm and extend this finding, we determined rs200538373 genotypes in a set of 57 cDNA samples generated from AD and non-AD brains; this effort identified a single sample that was heterozygous for rs200538373 (G/C) while the remainder were major allele (G/G) homozygous. The frequency of the rs200538373 minor C allele in our cohort was 0.8%, similar to that reported previously in European datasets [6, 28].

To explore the effects of the C allele of rs200538373, we sought to confirm whether aberrant splicing occurs in the brain of an individual with this allele. To assess splicing, cDNA from the rs200538373 heterozygous sample as well as several rs200538373 major allele homozygous samples was subjected to RT-PCR with amplicons spanning exon 40 to exon 42 (Figure 1A). We observed the expected product along with an additional longer amplicon for the sample that was heterozygous for rs200538373 (Figure 1B). Sanger sequencing of this longer amplicon found that exon 41 was extended 14 bp into the typical intron 41. Interestingly, the sequence electropherogram shows the rs200538373 minor C allele but not the major G allele (Figure 1C). Although not quantitative, this suggests that this abnormal splice form is largely produced from the minor allele of the *ABCA7* pre-mRNA. The 14 bp inclusion would encode an in-frame UGA termination codon beginning at position 2 in the extension and is therefore predicted to produce a truncated ABCA7 protein.

To test directly whether rs200538373 modulates exon 41 splicing, we generated minigenes for each *ABCA7* allele. The constructs included all of exon 41, intron 41, and exon 42 (Figure 2A). Since *ABCA7* is expressed in neurons, as well as other brain cell types [10, 29, 30], we tested the minigene splicing in the human neural cell line Be(2)-M17. We found that the transcript from the minigene with the rs200538373 minor C allele was spliced to generate exon 41 with the 14 bp extension (Figure 2B). Conversely, the minigene with the

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rs200538373 major G allele was spliced to generate exon 41 at the usual 5′ consensus splice site. The effect of the SNP in this assay was very robust, with minimal “normal” isoform generated by the minigene carrying the minor C allele and minimal extended isoform generated by the minigene carrying the major G allele.

To gain further insight into this finding, we used MaxEntScan::score5ss, a 5′ splice site prediction tool, to compare the normal 5′ consensus splice site in the presence of each rs200538373 allele and the aberrant splice site. We observe that the favorable score of the major G allele for rs200538373 at the normal splice site is reduced when the minor C allele of rs200538373 is present (Table 1). The aberrant splice site 14 bp further into the intron is unaffected by the SNP and has a splicing score that is competitive with the normal splice site when the G allele of rs200538373 is present. These results are consistent with rs200538373 being a functional SNP resulting in the normal 5′ consensus splice site being used when the G allele of rs200538373 is present and the aberrant splice site 14 bp further into the intron being used when minor C allele of rs200538373 is present.

Lastly, we assessed indirectly whether the aberrant splicing and predicted premature termination codon observed with the minor C allele of rs200538373 may be associated with nonsense mediated RNA decay by comparing *ABCA7* expression in rs200538373 major G allele homozygous individuals with that of the single rs200538373 heterozygous individual in our cohort of samples. For this effort, we established a quantitative model of *ABCA7* expression.. This model included ABCA7 expression as well as AD status and rs3764650, a common AD-associated ABCA7 SNP [3–5]. *ABCA7* expression was assessed previously by qPCR using primers in the constitutively spliced exons 44 and 45 [7] and those data are re-analyzed here. Since *ABCA7* is expressed in neurons and microglia, we compared models that evaluated *ABCA7* expression relative to expression of microglial and neuronal genes separately and together (Table 2). The *ABCA7* expression model that used only the neuronal mRNA synaptophysin had slightly more explanatory power (Figure 3A, adjusted R^2=0.700) than a model that included *synapthophysin* as well as microglial (*ITGAM* and *AIF1*) mRNAs (adjusted R^2=0.697). A model that used only the microglial mRNAs had a lower goodness-of-fit (adjusted R^2=0.323). Within each model, *ABCA7* expression was increased significantly in AD individuals and decreased significantly in carriers of the minor rs3764650 allele (Table 2, Figure 3A). We also analyzed these data by normalizing mRNA copy numbers to two “housekeeping” mRNAs (Figure 3B). Although this model is less visually striking (adjusted R^2 of 0.412), *ABCA7* expression was significantly increased in AD (standardized β coefficient=0.559, $p=3.0\times10^{-6}$) and the minor allele of rs3764650 was associated with a significant decrease in *ABCA7* expression (standardized β coefficient= −0.383, p=0.001). *ABCA7* expression in the individual that was heterozygous for rs200538373 was similar to that of other samples (Figure 3A–B, arrow). Hence, the individual with the minor C allele of rs200538373 did not have a large decrease in *ABCA7* expression, a finding we interpret as suggesting that nonsense mediated RNA decay does not grossly affect *ABCA7* expression. In summary, this quantitative analysis of *ABCA7* expression suggests that (i) the minor AD-protective allele of rs3764650 is associated with decreased *ABCA7* expression, (ii) AD itself is associated with increased *ABCA7* expression, and (iii) the rare AD-associated SNP rs200538373 acts through altered splicing

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without grossly affecting *ABCA7* expression levels, a finding supported by another quite recent study [31].

Discussion

The primary findings of this report are that abnormal *ABCA7* exon 41 splicing is found in the brain of an individual carrying the minor C allele of rs200538373 and that rs200538373 is a functional SNP. Additional findings include that rs200538373 does not appear associated with a gross decline in *ABCA7* expression, suggesting that NMD does not grossly impact *ABCA7* expression for this individual, and a confirmation that *ABCA7* expression is decreased with the minor allele of rs3764650 and increased in AD. As such, this report confirms and extends a report that rs200538373 was associated with *ABCA7* exon 41 splicing in blood [6] and our prior report that AD and rs3764650 was associated with expression per se [7].

The mechanisms underlying AD genetic risk factors are currently under intense investigation. Common *ABCA7* SNPs such as rs3764650 have been associated with a modest increase in AD risk (odds ratio: 1.23) while rare SNPs that involve premature stop termination codons, such as rs200538373, are associated with increased AD risk (odds ratio: 1.91) [4, 6, 32, 33]. In our original report examining rs3764650, we found that the minor allele of rs3764650 was associated with a decrease in *ABCA7* expression with a standardized beta-coefficient of −0.375 [7]. Here, we re-analyzed these *ABCA7* data by using a model that incorporated levels of mRNAs specific to microglia and neurons. This analysis produced a similar result, with the rs3764650 minor allele being associated with reduced *ABCA7* expression. Hence, modestly reduced *ABCA7* expression correlates with a modest increase in the AD odds ratio. In contrast, the rare rs200538373 was associated with aberrant exon 41 splicing that was predicted to produce a premature translation termination codon [6]. In pursuing the effect of this SNP, we observed a very robust SNP effect on exon 41 splicing *in vitro* and that the extended exon 41 isoform was clearly present in the brain of the rs200538373 minor allele carrier. That noted, the *in vivo* finding was limited because we identified only a single sample with the SNP minor allele. An additional limitation of this study was that we were unable to identify isoform specific qPCR primers and hence were not able to quantify the extended exon 41 splice variant. Our semi-quantitative gel-based analysis suggested that this extended exon 41 isoform was present at a level approaching that of the normal *ABCA7* isoform. Hence, the effect of the SNP may approach 50% of *ABCA7* mRNA containing a premature stop codon. This truncated ABCA7 protein would likely represent a loss of function because the encoded ABCA7 protein would lack its second ATPase domain. This effect could be greater if the truncated ABCA7 acts as a dominant negative; this possibility is supported by reports that similarly truncated ABCA1 proteins act as a dominant negative in dimer formation with full-length ABCA1 [34–36]. In summary, we interpret these results overall as showing that rs3764650, a common SNP associated with a modest reduction in *ABCA7* expression, leads to a small increase in AD risk. The increase in *ABCA7* observed in AD may represent a compensatory attempt to increase *ABCA7* and thereby reduce AD risk or may reflect other inflammation related processes. In contrast, rs200538373, a rare SNP associated with a more robust reduction in ABCA7, leads to a

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larger increase in AD risk. Overall, these results appear to essentially demonstrate a genetic dose response between functional impact and AD risk.

Considering the translational impact of these findings, agents that increase ABCA7 expression would be expected to reduce AD risk, especially for those individuals with the minor allele of rs3764650. Since ABCA7 expression is increased in AD, we speculate that a pharmacologic intervention to reduce AD risk would likely have to begin well before disease onset. With regards to rs200538373 and *ABCA7* splicing, we note that in this age of personalized medicine, several drugs that target splicing are in human trials or have been approved for human use. Some of these agents involve peripheral tissue, such as eteplirsen which targets splicing in a form of Duchene muscular dystrophy [37]. However, other agents target splicing in the central nervous system, with the drug nusinersen, which was recently FDA approved [38] for spinal muscular atrophy, being the most robust representative [39]. As science moves forward, agents that target aberrant splicing for AD may emerge.

In summary, our primary finding is an apparent dose response between the functional impact of AD-associated SNPs and AD risk. This finding is based upon (i) the observation that rs200538373, which is associated with robust AD risk, appears to have a robust effect on splicing *in vitro* with aberrant splicing found in the brain of a minor allele carrier and (ii) the observation that rs3764650, which is associated with modest AD risk, is associated with a modest reduction in *ABCA7* expression.

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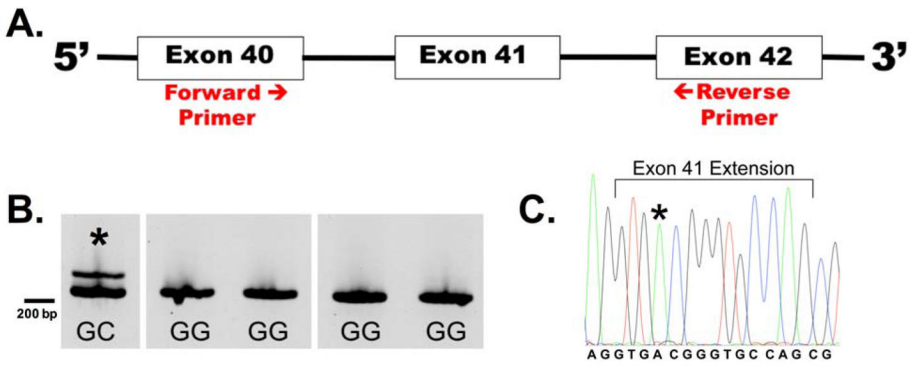

Figure 1.

Aberrant *ABCA7* exon 41 splicing in an rs200538373 heterozygous sample. A). RNA purified from the brains of a rs200538373G/C individual and several rs200538373G/G individuals was reverse transcribed and subjected to PCR with primers corresponding to sequences within exons 40 and 42. The amplicon size for normal splicing was 222 bp. B). The expected splice product as well as a longer *ABCA7* isoform was consistently detected only in the rs200538373G/C individual (denoted by *). C). Sanger sequencing of the longer *ABCA7* isoform found that exon 41 was extended by 14 bp relative to the expected isoform. This sequence from the rs200538373 heterozygous individual includes the last two bp of exon 41, the 14 bp extension of exon 41, and the first two bp of exon 42. Only the rs200538373 minor C allele is observed (marked by asterisk) (blue=C, black=G, green=A, red=T).

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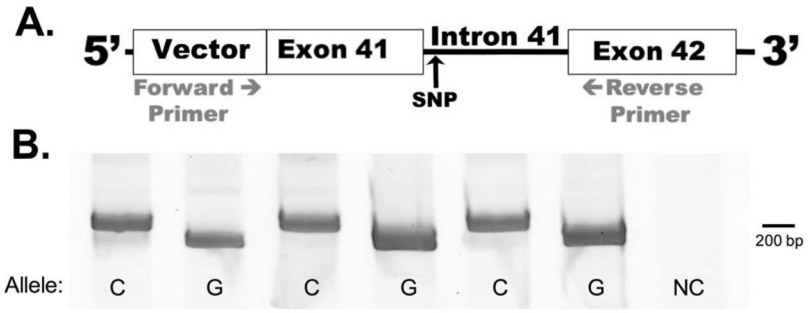

Figure 2.

Rs200538373 is a functional SNP that modulates *ABCA7* exon 41 splicing. A). Cells were transfected in triplicate with constructs that expressed ABCA7 exon 41, intron 41, and exon 42 and that carried with the rs200538373 major G or minor C allele. These transcripts also included vector-derived 5′ and 3′ sequence. B). RT-PCR analyses with a forward primer targeting vector sequence and a reverse primer targeting exon 42 detect normal splicing (192 bp amplicon) from the major G allele minigene and aberrant splicing (206 bp amplicon) in the minor C allele minigene. Note that 'C' and 'G' labels indicate rs200538373 alleles while 'NC' labels a negative control sample wherein cells were transfected in parallel with an irrelevant (GFP) gene. This result was replicated in three independent experiments.

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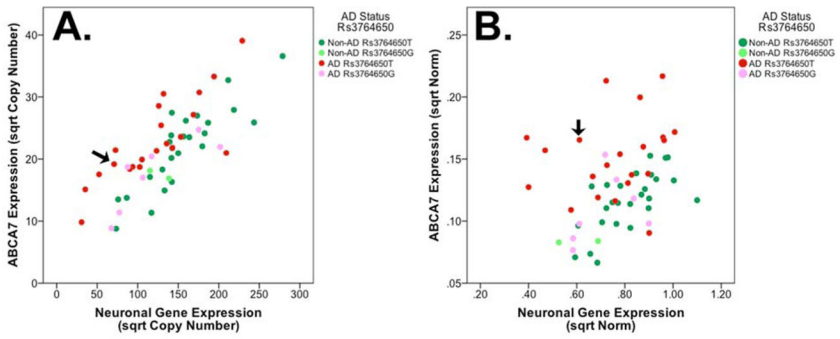

Figure 3.

ABCA7 expression is associated with AD status and rs3764650 but appears unaffected by rs200538373. A). When *ABCA7* expression was analyzed by comparing *ABAC7* mRNA copy numbers relative to a neuronal mRNA, expression appeared to correlate with rs3764650 and AD status. For both A and B, the arrow points to the single sample that was heterozygous for rs200538373. Additionally, rs3764650T refers to rs3764650T/T homozygous samples while rs3764650G refers to rs3764650G/T heterozygous samples as well as a single rs3764650G/G homozygous sample. B). *ABCA7* expression relative to AD status and rs3764650 when mRNA copy numbers were normalized to *RPL32* and *EIF4H* housekeeping mRNAs. For both A and B, the square root of values was used to generate a normal distribution of the data.

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Table 1

Impact of rs200538373 on *ABCA7* exon 41 splicing. This SNP is at position 5, in parenthesis, within intron 41 and is predicted to weaken the splice site, i.e., the prototypic splice donor sequence is CAG/gtragt (exon in upper case, intron in lower case, r = purine).

Exon 41 Splicing	Splice Site Sequence (splice site at ^)	Splicing Score
Normal Splice Site Major G allele	CAG^gtga(g)g	10.07
Normal Splice Site Minor C allele	CAG^gtga(c)gggtgccaggtaggg	7.66
Aberrant Splice Site Extended Exon 41	CAGgtga(g/c)gggtgccag^gtaggg	9.46

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Table 2

Evaluation of cell-type specific reference gene expression in *ABCA7* expression model. The neuronal reference gene was *synaptophysin* while the microglial reference was the geometric mean of *ITGAM* and *AIF1* expression. The model with both cell types used the geometric mean of *synaptophysin* and the microglial geometric mean.

Reference Gene Type	Adjusted R^2	AD Beta Coefficient (p value)	Rs3764650 Beta Coefficient (p value)	Reference Gene Beta Coefficient (p value)
Neuronal	0.700	0.322 (1.7×10^{-4})	−0.236 (0.003)	0.840 (7.1×10^{-15})
Microglial	0.323	0.240 (0.050)	−0.257 (0.029)	0.556 (1.6×10^{-5})
Both Cell Type	0.697	0.337 (3.9×10^{-4})	−0.234 (0.008)	0.805 (1.6×10^{-12})