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PRELIMINARY DEMONSTRATION OF AN ALLELIC ASSOCIATION OF THE *IREB2* GENE WITH ALZHEIMER'S DISEASE

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Abstract

The role of iron metabolism in Alzheimer's Disease (AD) is well documented. Regulation of the proteins that maintain cellular iron metabolism is mediated by two cytoplasmic RNA-binding proteins, the Iron Regulatory Proteins (IRP1 and IRP2), that function through post-transcriptional interactions with RNA stem loop structures called iron-responsive elements. As the primary mediator of iron homeostasis in neuronal cells, IRP2 is a strong candidate for polymorphisms that could impact AD pathogenesis. Thus, we performed a pilot study to assess polymorphisms in the gene encoding IRP2 (*IREB2*) on clinically well-characterized, postmortem samples (50 AD and 50 controls). DNA sequence analysis of the *IREB2* gene region revealed 14 polymorphisms. Two (rs2656070 and rs13180) showed statistically significant skewing of allelic and genotypic distributions between AD patients and controls. *In silico* analyses revealed that rs2656070 lies within a probable promoter and disrupts the binding sites of at least two known transcription factors. Though silent and likely not functionally relevant, rs13180 is in complete LD with rs2656070 ($D' > 0.999$), creating an *IREB2*-haplotype that is significantly associated with AD. Confirmation of this association in a larger cohort of cases and controls would further support the role of iron regulation in the pathogenesis of this catastrophic and increasingly common neurodegenerative disorder.

Keywords

Iron; Iron Regulatory Protein 2; Neurodegeneration; Neurodegenerative Disease; Iron metabolism; Alzheimer's disease

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of disabling cognitive impairment in older persons. According to one estimate, this disorder afflicts about 10% of everyone over the age of 65 and almost half of everyone over the age of 85 [12]. As people live longer, the number of afflicted individuals is projected to multiply. Clinically, AD is characterized by a gradual but progressive impairment in memory and other cognitive domains. Neuropathologically, the disorder is characterized by neuritic plaques, neurofibrillary tangles, other histopathological features, and a loss of synapses and neurons [27]. Although it has been demonstrated that iron accumulates in brain regions which are histopathologically affected by AD [29], it remains unclear whether this accumulation is a cause or consequence of these histopathological features.

Although iron is essential to cellular function, dysregulation of iron metabolism can be toxic to cells. The regulation of the proteins that maintain iron homeostasis is mediated by a pair of cytoplasmic RNA-binding proteins known as iron regulatory proteins (IRP1 and IRP2) [25]. These proteins function through post-transcriptional interactions with RNA stem loop structures called iron-responsive elements (IREs) [13]. Abnormal accumulation of iron in the brain has been implicated in a variety of neurodegenerative diseases, most notably AD [9].

The genetics of AD remain complex and obscure despite intensive research. Early onset familial forms of AD appear to be related to mutations in three genes with high penetrance, the β-amyloid precursor protein (AβPP) gene on chromosome 21 [6], the presenilin 1 (PSEN1) on chromosome 14 [17,28], and the presenilin 2 (PSEN2) gene on chromosome 1 [17,28], all resulting in increased amyloid beta (Aβ) production and subsequent neurodegeneration. However, extensive research has demonstrated that less than 5% of all cases of AD can be traced to these genetic factors. On the contrary, the majority of AD cases are sporadic, being associated with late onset and complex genetic etiology. The greatest genetic risk factor associated with this sporadic form of AD is the apolipoprotein E (APOE) ε4 allele on chromosome 19 [11], however, at least 30 additional candidate genes coding for proteins and processes considered critical for disease pathogenesis (oxidative stress, apoptosis, inflammation, increased expression of Aβ, etc.) have been identified, though not consistently confirmed [8].

Several lines of evidence link dysfunctional iron regulation to the known AD-associated genetic factors including the ability of iron to regulate translation of the AβPP transcript through its association with a novel IRE motif in the 5′ upstream region of the AβPP transcript [24] as well as post-translationally modulate the α-secretase cleavage of AβPP itself [5]. Though the precise makeup of α-secretase has yet to be determined, γ-Secretase-mediated cleavage of AβPP has been shown to be mediated by the presenilin genes [14]. The APOE gene has been linked to regulation of cholesterol [15], which has been implicated in promoting iron-dependent oxidative damage in neurodegeneration [20] and iron has been shown to increase Aβ aggregation in an APOE isoform-dependent manner [18]. Another iron-related susceptibility factor that appears to be correlated with AD pathogenesis, and exacerbated in APOE4 carriers, is the coexpression of the C2 allele of transferrin (TF) gene with the C282Y allele of the hemachromatosis (HFE) gene [23]. An additional line of evidence demonstrating the role of iron in AD pathogenesis is the fact that iron chelators significantly enhance the solubilization of Aβ deposits from postmortem AD brain samples [7]. In fact, the metal chelator Clioquinol has been demonstrated to slow the clinical progression of AD in humans [22].

Thus, iron dysregulation is viewed as an increasingly important factor in the etiology of AD and as the primary mediator of iron homeostasis in neuronal cells, IRP2 is a strong potential candidate for polymorphisms that could play a role in AD. The gene encoding IRP2 (*IREB2*)

is located on chromosome 15q25.1 and lies 12.5 Mbp and 23.5 Mbp from previously identified AD-associated linkage peaks at 15q22 [26] and 15q26 [4], respectively. Ablation of the *IREB2* gene results in neurodegenerative disease in mice via a mechanism of iron dysregulation [16]. Lastly, IRP2 displays a significantly different distribution between normal and AD-affected brains and has been co-localized with redox-active iron to histopathological features of AD including neurofibrillary tangles, senile plaques, and neuropil threads [29].

In order to determine whether or not polymorphisms in the *IREB2* gene are associated with and potentially pathogenic in AD, we performed a pilot study to screen the entire *IREB2* gene in 100 clinically well-characterized, post-mortem brain samples (50 AD patients and 50 unaffected controls) for sequence polymorphisms that are significantly associated with AD. We report here the identification of a statistically significant association between an *IREB2* haplotype and the AD phenotype.

MATERIALS AND METHODS

Population samples

Fresh frozen brain tissue specimens (~500 mg) from the occipital lobe of 100 brain donors were obtained from the Human Brain and Spinal Fluid Resource Center (Los Angeles, CA). The donors included 50 persons who satisfied the most recent CERAD criteria for the neuropathological diagnosis of AD [1,19], including presence of both neuritic plaques and neurofibrillary tangles in the neocortex (i.e. frequent neuritic plaque score according to CERAD) and 50 persons who did not satisfy these criteria (i.e. neuropathological examination confirmed the absence to pathological changes diagnostic for AD). The AD cases were 83 ± 7.0 years of age at the time of death and included 31 females and 19 males. The non-AD controls were 58 ± 20.9 years of age at the time of death and included 15 females and 35 males. The non-AD controls showed no evidence of histopathological features associated with AD or dementia (according to their last antemortem assessment). The sample population was predominantly Caucasian (86%), but included individuals of African (10%), Latino (2%), Asian (1%), and Native American (1%) descent (see Table 1 for a breakdown of demographic characteristics).

Isolation, amplification, and sequencing of DNA

Genomic DNA was extracted from the fresh frozen brain specimens using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) according to manufacturer's specifications. Following isolation of DNA from all 100 of the fresh frozen brain samples, the DNA was quantitated using the PicoGreen dsDNA quantitation system (Molecular Probes Inc., Eugene, OR), and diluted to 10 ng/μL. 50 ng of total genomic DNA was used to amplify each of the 22 exons and the 2,241 bp of the 5′ upstream region (purported to contain the *IREB2* promoter region) of the *IREB2* gene using the primers listed in Table 2 and standard PCR protocols (available upon request). The entire 2,241 bp of the 5′ upstream region was amplified using the promoter region 1 forward primer (5′ CCTGCCTCTGTCTCACGGTA 3′) and the promoter region 4 reverse primer (5′ AACTGCCAGACCCAGCTCGGA 3′), whereas the 22 exons were amplified individually (including ~50–100 bp of flanking sequence containing intron/exon spicing junctions), with the exception of exons 17 and 18 and exons 19 and 20, which were amplified in tandem due to their close proximity (but still sequencing 5′ and 3′ splice junction sites). All of the primers used in exon amplification contained either the M13 (−21) universal forward tail (5′ TGTAAAACGACGGCCAGT 3′) or the M13 universal reverse tail (5′ CAGGAAACAGCTATGACC 3′). PCR products were visualized by agarose gel electrophoresis.

Amplified PCR templates were purified with Montage Multiscreen $PCR_{\mu 96}$ filter plates (Millipore Inc., Billerica, MA) according to the manufacturer's specifications and resuspended in 25 μL molecular biology grade (MBG) water. The purified samples were analyzed by agarose gel electrophoresis and compared to a known standard to determine the optimum concentration for DNA sequence analysis.

For the 22 exons, both strands of each PCR product were sequenced as follows: Sequencing reactions were performed using 3 μL (approximately 25 ng) of purified PCR product in a 6 μL reaction containing 0.33 μL BigDye Terminator v3.1 premix (Applied Biosystems, Foster City, CA), 3.2 pmol of either M13 forward or M13 reverse primer, and 1.03 μL 5X BigDye sequencing buffer. Cycle-sequencing was performed for 35 cycles following the manufacturers recommendations on GeneAmp 9700 PCR machines (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using CleanSEQ® (Agencourt Biosciences Corp., Beverly, MA) to remove unincorporated dye-terminators, and analyzed on 3730xl DNA analyzers (Applied Biosystems, Foster City, CA). The 5′ upstream region was sequenced in four segments using the purified 2,241 bp amplicon as the template and 4 sets of internal sequencing primers as shown in Table 2. Otherwise, the protocol was identical to that above.

Sequence and statistical analyses

DNA sequences were checked for accuracy by sequencing both strands in opposite directions and comparing the sequence of each strand to one another. Individual forward and reverse sequence traces were aligned to each other in the *Sequencher* program (Gene Codes Corp., Ann Arbor, MI) and ambiguities were resolved by visual observation of peak heights. Polymorphisms in exons were identified by comparison to the updated reference sequence for the *IREB2* cDNA in the GenBank database (NCBI accession NM_004136), whereas polymorphisms in the 5′ upstream region were identified by comparison to an available genomic reference sequence for *IREB2* in the Celera database (Celera accession hCG38938). The novelty of the observed polymorphisms was assessed by screening the available SNP database (http://genome.ucsc.edu/) for previously demonstrated occurrences of the SNP.

The distribution of sequence polymorphisms between affected and unaffected controls was assessed and statistically evaluated via the chi-square test. Those found to be statistically significant were additionally tested using the Fisher's Exact statistical test as well as the Bonferonni correction for multiple testing. Analysis of linkage disequilibrium, haplotype reconstruction, and haplotype trend regression analysis was done with PowerMarker v. 3.22 (http://statgen.ncsu.edu/powermarker/). Polymorphisms demonstrating statistically significant distributions between AD patients and unaffected controls were subject to further analyses of functional relevance via a variety of *in silico* tests.

In silico analyses

In order to assess potential functional relevance of SNPs located in the 5′ upstream region of the *IREB2* gene, *in silico* promoter analysis was performed on the 10,000 bp of 5′ upstream region flanking sequence using the NIH promoter scanning software package PROSCAN v. 1.7 (http://bimas.cit.nih.gov/molbio/proscan/) [21]. The 500 bp sequence containing the putative promoter region was subsequently used to search the TRANSFAC 6.0 database of transcription factor binding sites (http://www.gene-regulation.com/pub/databases.html#transfac) via the Ali Baba v. 2.1 software package. SNP rs13180 was tested for its ability to act as an exonic splice enhancer using the ESE Finder (http://rulai.cshl.edu/tools/ESE/).

RESULTS

Sequence analysis of the entire *IREB2* gene (including intron/exon splice sites) as well as 2,241 bp of the 5′ upstream region identified 14 polymorphisms (Table 3). Five of the polymorphisms were located in coding regions, one was in an intronic region between exons 19 and 20, and the remaining eight were found in the 5′ upstream region. There were ten transitions and four transversions. All of the polymorphisms were blasted using blastn [2] against the HG16 build (NCBI Build 34) of the UCSC Genome Browser (http://genome.ucsc.edu/) SNP track on Oct. 27th, 2004. All numbering is from the initiator methionine ATG (A = 1) based on the *IREB2* cDNA sequence (NCBI accession NM_004136) and/or the *IREB2* genomic sequence (Celera accession hCG38938), which are noted as the "reference" sequence. Six of the polymorphisms have been previously identified and assigned refseq numbers (bp –2072 A/G = rs2102264; bp –763 C/G = rs2568484; bp –428 G/A = rs2656070; bp –89 T/C = rs954144; X19+12 C/A = rs4887060; and bp 2616 C/T = rs13180). The other eight polymorphisms have not previously been described. Three of the polymorphisms located within exons changed an amino acid, whereas the other two were silent. Each of the 14 identified polymorphisms was tested individually for differences in genotype distribution between groups (AD vs. unaffected control). Using the chi-square test, two of the polymorphisms (rs2656070 and rs13180) demonstrated a statistically significant genotype distribution between AD patients and unaffected controls at the $p < 0.05$ and $p < 0.001$ levels, respectively, with no correction for type 1 errors (Table 3). These two SNPs were further tested using the Fisher's Exact test and found to demonstrate statistical significance at the $p < 0.035$ and $p < 0.001$ levels, respectively, with no correction for Type 1 errors. Applying the Bonferonni correction for multiple testing (*alpha' = 0.05/14 = 0.0036*), rs2656070 fails to maintain statistical significance ($p > 0.004$), but rs13180 retains its statistical significance ($p < 0.001$). The precise genotypic distribution of these two significant SNPs is displayed in Table 4.

The bp –428 G/A polymorphism in the 5′ upstream region of the *IREB2* gene (rs2656070) has been observed previously, but no allele frequency information is currently available. In order to assess whether or not this polymorphism has any functional relevance, an *in silico* promoter analysis was performed whereby 10,000 bp of 5′ upstream region sequence from the *IREB2* gene was analyzed using the NIH promoter scanning software package PROSCAN v. 1.7 (http://bimas.cit.nih.gov/molbio/proscan/) [21]. The highest scoring predicted promoter region (promoter score: 83.68) was located between bp 9,502 and p9,752 in the forward strand, which corresponds to between –248 and –498 bp upstream of the transcriptional start site (bp –428 G/A lies within this region). A 500 bp sequence containing this putative promoter region was subsequently used to search the TRANSFAC 6.0 database of transcription factor binding sites (http://www.gene-regulation.com/pub/databases.html#transfac) via the Ali Baba v. 2.1 software package. The results of this analyses showed that rs2656070 disrupts the recognition sequence of at least two known transcription factors (AP-1 and Sp1). Thus, *in silico* analyses suggest that rs2656070 lies within the most highly predicted promoter sequence in the *IREB2* gene and disrupts the recognition sequence of at least two known transcription factors.

The bp 2616 C/T polymorphism is silent, maintaining an alanine at amino acid position 872. As mentioned, this polymorphism has also been described previously (rs13180) and the frequency of the C/T allele is 0.564 and 0.436, respectively (http://www.ncbi.nlm.nih.gov/SNP/). This SNP was tested for the ability to act as an exonic splice enhancer (ESE) using ESE finder (http://rulai.cshl.edu/tools/ESE/). This polymorphism actually demonstrates the ability to act as an ESE, but the base change from C to T doesn't appear to significantly affect this potential.

Due to the high level of statistical significance for this polymorphism observed between AD patients and unaffected controls, we predicted that it may be linked to another polymorphism

that is the true functional variant. To identify the extent of LD from the significantly associated SNPs, we calculated pairwise linkage disequilibrium statistics for all observed polymorphisms using the PowerMarker v. 3.22 program (http://statgen.ncsu.edu/powermarker). The two statistically significant SNPs (rs2656070 and rs13180) were found to be in almost complete linkage disequilibrium (D′ > 0.999) creating an *IREB2*-related haplotype. Haplotype trend regression analysis revealed that this haplotype is significantly associated with AD risk ($p < 0.001$). Because the SNPs are interrelated, we used forward and backward logistic regression analysis with both SNPs as independent variables and using the additive model. Under this model, only rs13180 remained significant ($p < 0.001$, OR 4.4, chi-square = 10.9). No significance remained for rs2656070 ($p = 0.9$).

DISCUSSION

In this preliminary study of 50 neuropathologically well-characterized AD cases and 50 unaffected controls, AD was significantly associated with an *IREB2*-related haplotype with a potentially functionally relevant SNP in the 5′ upstream region ($p < 0.001$). DNA sequence analysis of all 22 exons of the *IREB2* gene (including splice junction sites) as well as 2,241 bp of the 5′ upstream region containing the promoter revealed the presence of 14 polymorphisms. Two of these, a G/A transition at bp –428 in the 5′ upstream region (rs2656070) and a silent C/T transition at bp 2616 in exon 21 of the *IREB2* coding region (rs13180), showed statistically significant distributions between AD patients and unaffected controls via chi-square at the $p < 0.05$ and $p < 0.001$ levels ($p < 0.035$ and $p < 0.001$ via Fisher's Exact test), respectively.

In silico analyses revealed rs2656070 lies within a probable promoter and disrupts the binding sites of at least two known transcription factors (AP-1 and Sp1). Although c-MYC has been suggested to play a role in the regulation of *IREB2* transcription [31], a clear understanding of *IREB2* transcriptional regulation remains elusive. Thus, the finding that rs2656070 is located in a probable promoter region and disrupts the binding sites of these two different transcription factors is intriguing and warrants further investigation. Though silent and likely not functionally relevant despite a potential role as an exonic splice enhancer, rs13180 shares significant levels of linkage disequilibrium with rs2656070 (D′ > 0.999), creating an *IREB2*-related haplotype that is significantly associated with AD ($p < 0.001$). These results suggest that there have been minimal amounts of historic recombination in the *IREB2* genomic region and that there is a strong correlation of alleles at these two sites. Interestingly, rs13180 is located at the very end of a haplotype block in Caucasians (http://www.celera.com). Also interestingly, this haplotype block ends with rs13180 only in Caucasians, whereas Chinese and African populations display an extended block that includes the nearby locus LOC123688. It is also possible that rs13180 is also in LD with an as yet unidentified SNP and it is known that a group of nicotinic acetylcholine receptor subunits known to be associated with AD [10,30] are located about 200 kb 3′ upstream of rs13180 [3].

Although the finding of the statistically significant polymorphisms with *in silico* data suggesting a possible functional role for the SNP is intriguing, findings from our study must be considered exploratory, given the small number of subjects and potential for Type 1 errors. Type I errors are essentially "false positives" that occur when multiple tests are performed, whereby statistical associations are seen purely by chance alone. Using a confidence interval of 95% ($p < 0.05$), the chances of obtaining a statistically significant association by chance alone is 1 in 20 (5%). That chance increases to 7% in our sample due to the smaller number of tests (i.e. 14 SNPs/tests). To guard against Type 1 errors caused by multiple testing, we applied the Bonferroni correction (dividing the confidence interval by the number of tests) to the two SNPs shown to be significant and found that only rs13180 retains statistical significance ($p < 0.001$).

It should also be noted that the cases and controls show some disparity in mean age. In order to determine whether or not age had any effect on our genotypic and allelic distributions, we reran the logistic regression analysis, a regression-based analysis testing the association of haplotype and trait (quantitative or qualitative) with one regression coefficient per haplotype permitting the inclusion of covariates and moderator variables (e.g. age, gender, etc), adjusting for age and gender and found that the haplotype remains statistically significant ($p = 0.043$). To further rule out the possibility that our association is due to solely to age, we removed all young individuals (< 65 yrs. old) from the control cohort and re-analyzed the data (n = 21). Using this model and the Fisher's Exact test, we find that the significance of the two SNPs is maintained, albeit at lower levels ($p < 0.033$ for rs2656070 and $p < 0.034$ for rs13180), due to reduced power.

Because the two significant SNPs are interrelated (D′ = 0.99), we ran forward and backward logistic regression analysis with both SNPs as independent variables using the additive model. This analysis revealed that only rs13180 remained significant at the $p < 0.001$ level (OR 4.4, chi-square = 10.9) and no significance remained for rs2656070 ($p = 0.9$), which is expected as rs13180 seems to be driving the association. However, given that LD does not always decay in a linear mode, the analysis of promoter SNPs (i.e. rs2656070) could still be promising. If non-Caucasians are excluded, the picture is the same at the $p < 0.004$ level due to slightly reduced power. Additionally, in order to further exclude the possibility that our distributions were skewed by ethnicity, we eliminated the 10 Africans, 2 Latinos, 1 Asian, and 1 Native American present in the sample and re-analyzed the data. The statistical significance of the distributions of these two SNPs either increased or remained unchanged with the exclusion of samples that could possibly impose any racial bias.

In conclusion, we find by application of multiple statistical models, that the association of the driving SNP (rs13180) as well as the haplotype to the AD phenotype remains statistically significant despite any potential age and/or racial bias. Though extremely encouraging, these findings need to be confirmed in an independent sample and their functional significance clarified in additional validation experiments. In order to assess the frequency of these polymorphisms/haplotype more precisely, we propose to genotype these SNPs in larger samples of cases and controls. Once haplotype frequencies are established, we propose to determine the functional relevance of this haplotype by cloning haplotype-specific promoter fragments into the pGL3-Basic vector and assaying promoter activity via a dual-luciferase system. If the association between this haplotype and AD can be confirmed in a large, ethnically diverse population, it would provide further support for the role of iron in the pathogenesis of AD and provide additional information about the heritable risk factors associated with this catastrophic and increasingly common neurodegenerative disorder.

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