Unraveling the biological mechanisms in Alzheimer's disease – lessons from genomics

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Abstract

Alzheimer's disease (AD) is the most common form of dementia and the most common neurodegenerative disease, with a complex genetic background. Genome wide association studies (GWAS) have yielded important new insights into genetic mechanisms of AD pathology. Current results unequivocally confirm apolipoprotein E (APOE) as a major genetic risk factor for development of late onset AD. Additional associations of more than twenty genes have also been identified and replicated in subsequent genetic studies. Despite the exciting new GWAS data which have emerged in the last few years, it has become clear that common variants within the genome cannot fully explain the underlying genetic risk for AD. Novel approaches such as genome-wide analysis of copy number variations (CNV) or low-frequency rare functional gene variants may provide additional insight into genetic basis of AD. In this review we summarize the findings of eighteen GWAS studies in AD performed to date, with an emphasis on potential future developments in the quest for genetic risk factors of AD.

Keywords: Alzheimer’s disease, genomics, genome-wide association studies, copy number variants

List of abbreviations: Aβ, β-amyloid; ACAN, aggrecan; ACE, angiotensin I converting enzyme; AD, Alzheimer's disease; APOE, apolipoprotein E; APP, amyloid precursor protein; ARSB, Arylsulfatase B; ATXN1, ataxin 1; BCR, breakpoint cluster region; BIN1, bridging integrator 1; BLOC1S3, biogenesis of lysosomal organelles complex-1, subunit 3; CAND1, cullin-associated and neddylation-dissociated 1; CH25H, cholesterol 25-hydroxylase; CHRNA7, cholinergic receptor, nicotinic, alpha 7; CHRNB2, cholinergic receptor, nicotinic, beta 2; CHARGE, Cohort for Heart and Aging Research in Genomics Epidemiology; CLU, clusterin; CNV, copy number variations; CNTN5, contactin 5; CR1, complement component (3b/4b) receptor 1; CST3, cystatin C; CTSS, cathepsin S; DISC1, disrupted in schizophrenia 1; ECT, entorhinal cortex thickness; EBF3, early B-cell factor 3; EFNA5, ephrin-A5; FAM113B, family with sequence similarity 113, member B; FAM63A, family with sequence similarity 63, member A; FDR, false discovery rate; GAB2, GRB2-associated binding protein 2; GALP, Galanine and galanine-like peptides; GRB2, growth factor receptor bound protein;; GWAS, Genome wide association studies; KEGG, Kyoto Encyclopedia of Genes and Genomes;; LD, linkage disequilibrium; LMNA, , lamin A/C; LRAT, lecithin retinol acyltransferase; MAGI2, membrane associated guanylate kinase, WW and PDZ domain containing 2; MAPT, microtubule-associated protein tau; MARK4, MAP/microtubule affinity-regulating kinase 4; MRI, magnetic resonance imaging; MYH13, myosin, heavy chain 13; MTHFD1L, methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like; PCK1, phosphoenolpyruvate carboxykinase 1; PCDH11X, protocadherin 11 X-linked; PGBD1, piggyBac transposable element derived 1; PICALM, phosphatidylinositol binding clathrin assembly protein; PRNP, prion protein; PRUNE2, prune homolog 2; PSEN, presenilin; QT, quantitative trait; RNF220, ring finger protein 220; SNP, single nucleotide polymorphism; SORL1, sortilin-related receptor; TF, transferrin; THF, tetrahydrofolate; TNK1, tyrosine kinase, non receptor 1; TOMM40, translocase of outer mitochondrial membrane 40 homolog; TPT, temporal pole cortex thickness; TRAK1, trafficking protein, kinesin binding 1; TRPC4AP, transient receptor potential cation channel, subfamily C, member 4 associated protein; UBD, ubiquitin D; UTP20, UTP20, small subunit (SSU) processome component, homolog; WML, white matter lesion volume; ZNF224, zinc finger protein 224

Introduction

Alzheimer's disease (AD) is the most common form of dementia and the most common neurodegenerative disease. The estimated prevalence of the disease in 2006 was approximately 27 million people, with the highest number of patients present in the regions of Asia and Europe (Brookmeyer et al., 2007). The number of patients with dementia is increasing by roughly 4.5 million people annually (Ferri et al., 2005), and is projected to reach more than 115 million by the year 2050 (Prince and Jackson 2009).

The disease is characterized histopathologically by the accumulation of β-amyloid plaques and neurofibrillary tangles, leading to progressive neuronal and synaptic loss in the limbic and association areas of cortex and in subcortical nuclei (Selkoe 1991; Chertkow et al., 2001; Tiraboschi et al., 2004; Takashima, 2009). The AD patients exhibit a plethora of symptoms related chiefly to progressive cognitive and functional decline (Waldemar et al., 2007). The pathogenesis of AD still remains not fully elucidated, but deposition of β-amyloid (Aβ) 1-42 derived from amyloid precursor protein (APP), appears to be a key element contributing to oxidative stress, tau pathology, mitochondrial insufficiency and synaptic failure. Aβ, which is the major component of senile plaques, is cleaved from APP first by β-secretase, and then by the γ-secretase.

Genetic background of the disease is heterogeneous and complex, without a straightforward mode of inheritance. The patients can be divided in two major forms of the disease, namely those with an early age of onset, usually below 65 years of age, and patients with the late onset AD (LOAD), typically well beyond 65 years. The patients with early onset of the disease show Mendelian transmission and are caused

by mutations in three genes, which are all involved in the production of Aβ (Tanzi and Bertram, 2005). The mutations affect the APP (Goate et al., 1991), presenilin 1 (PSEN1; Sherrington et al.,1995) and presenlin 2 (PSEN 2; Levy-Lahad et al., 1995; Rogaev et al., 1995) genes, interfering with the normal cleavage of APP by the γ-secretase complex.

Overwhelming majority of AD patients exhibit the late onset of the disease and show less-obvious familial aggregation. Despite strong evidence of heritability (Bergem et al., 1997; Gatz et al., 2006), genetic mechanisms involved in late onset AD have been much more difficult to elucidate. The disease pathophysiology in these patients is most likely linked to a whole set of susceptibility genes affecting various pathways, as including those involved in Aβ production, such as SORL1, GAB2 or CH25H (Andersen et al., 2005; Zerbinatti et al., 2008), aggregation, such as CST3 or PRNP (Kaeser et al., 2007; Schwarze-Eicker et al., 2005), and clearance, such as ACE (Bertarm and Tanzi, 2009; Sleegers et al., 2010). The role of several other susceptibility genes has also been implicated in other pathophysiological pathways, such as TF, MAPT and GAB2 in oxidative stress (Yamamoto et al., 2002; Ballatore et al., 2007; Nizzari et al., 2007), CHRNB2 in Ach transmission (Oddo et al., 2006), CR1 and CLU in inflammation damage (Khera and Das, 2009; Zanjani et al., 2005) or PICALM in intracellular trafficking of synaptic vesicle proteins (Harel et al., 2008). Over past several decades, more than 500 genes have been associated with increased risk of AD, mainly by utilizing the candidate-gene approach (Bertram and Tanzi, 2008).

Despite the large number of candidate genes, only a few have been reproducibly shown to influence disease risk or onset age (Bertram et al., 2007). Among those genes, the ε4-allele of the apolipoprotein E gene (APOE) has shown the strongest

risk effect for the development of AD (Strittmatter et al., 1993; Saunders et al., 1993). APOE is found within senile plaques (Namba et al., 1991), binds Aβ (Strittmatter et al., 1993), may influence neuritic formation of plaques in mouse models of the disease (Holtzman et al., 2000) and is involved in Aβ deposition and clearance in the brain (Holtzman, 2004). Despite the strong evidence for its role in disease pathophysiology, APOE as a genetic risk factor is not fully penetrant, and is neither necessary not sufficient for the development of AD (Ertekin-Taner, 2010). Heterozygous carriers of the ε4 genotype exhibit a two to four times greater AD odds ratio when compared to homozygous ε3 carriers. In homozygous ε4 carriers the odds ratio increases to 6 to 30, as shown in population-based studies in subjects of European origins (Ertekin-Taner, 2007). However, the effect of APOE ε4 seems to be age dependent, and its use as a diagnostic and predictive factor in a clinical setting is not feasible (Knopman et al., 2001).

Deciphering of the human genome and development of high-throughput genomic technologies (Manolio and Collins, 2009) have accelerated the efforts aimed unraveling of underlying pathophysiological mechanisms involved in AD. These achievements opened up to major avenues of scientific effort in research of AD. A new impetus was given to the search for candidate genes associated with increased risk of AD especially through performing genome-wide association studies (GWAS). In this review we give a comprehensive overview of the GWAS studies in AD performed so far, providing a novel overview of several GWAS approaches, from case-control studies, to copy number variations (CNV) analysis to quantitative-trait association studies. Furthermore, we also give emphasis on novel avenues of research, such as genome-wide analysis of CNV or low-frequency rare functional gene variants.

Genome-wide association studies in AD

Detailed identification and characterization of single-nucleotide polymorphisms (SNPs) in the human genome (Sachidanandam et al., 2001) and development of novel high-throughput SNP genotyping technologies enabled a more comprehensive insight into the genetic basis of common, complex diseases. In contrast to candidate gene studies, which do not allow results beyond the scope of the initial hypothesis, GWAS allow for simultaneous testing of a very large number of genetic markers. The studies usually involve analysis of tens of thousands of genetic markers in thousands of individuals, in a mostly hypothesis-free manner (Betram and Tanzi, 2009). The genetic markers utilized in the GWAS consist of SNPs chosen based on their ability to cover common variation in the human genome (McCarthy et al., 2008). More recent microarray technology also allows for assessment of CNVs, namely deletions or multiplications of genomic DNA at certain chromosomal regions.

Implementation of the GWAS approach has yielded a large number of genome-wide significant and replicated findings in many genetically complex diseases (Ioannidis et al., 2009). However it has been shown that most common variants individually or in combination confer relatively small increments in risk (1.1-1.5-fold) and explain only a small proportion of heritability (Hindorff et al., 2010). GWAS in AD have so far yielded much less reproducible results, when compared to studies of other complex diseases, with the exception of the APOE locus, whose association with AD was identified in all but one study, and always found to be orders of magnitude more significant than any of the newly implicated genes to date (Bertram and Tanzi, 2009).

Collective data overview and systematic meta-analysis of the association studies carried out in AD can be found at the AlzGene website. AlzGene represents the most comprehensive electronic database of the genetic association studies published in the field of AD, including GWAS (Bertram et al., 2007). The overview of the late onset AD GWAS results can be found in the Table 1.

The study of Grupe et al. 2007

The first published GWAS of late onset AD used a select set of more than 17,000 SNPs from 11,211 genes, chosen according to likelihood of being functional polymorphisms (Grupe et al., 2007). In the first stage of the study, genotyping was performed on a screening sample consisting of 380 AD cases and 396 control subjects from UK. Follow-up studies of the promising markers were performed in four independent cohorts from UK and USA, totaling more than 3000 subjects. Among the loci identified, APOE-related SNPs were the only ones to exhibit genome-wide significance. Besides APOE, 16 more loci were identified as nominally significant, namely ACAN, BCR, CTSS, EBF3, FAM63A, GALP, GWA_14q32.13, GWA_7p15.2, LMNA, LOC651924, MYH13, PCK1, PGBD1, TNK1, TRAK1 and UBD. Although none of these replicated in more than two of the five tested samples groups, four SNPs were especially interesting, namely GALP, TNK1, PCK1 and GWA_14q32.13, exhibiting significant p-values across all samples. Subsequent replication studies have shown mixed results regarding confirmation of the identified loci, with GWA_14q32.13, TNK1 and GALP showing the strongest association. Galanine and galanine-like peptides (GALP) have been implicated in inhibition of long term potentiation in the hippocampus, as well in suppression of cholinergic neurotransmission (Lang et al., 2007), and have been shown to be overexpressed in

the AD brains. On the other hand, tyrosine kinase, non receptor 1 (TNK1) has been shown to enable tumor necrosis factor α induced necrosis, providing a possible mechanisms for increased neuronal death in AD patients.

The studies of Coon et al. 2007 & Reiman et al. 2007

The second study utilized the approach of genotyping more than 500,000 SNPS on the Affymetrix 500K platform (Coon et al., 2007). The analysis was performed on a sample of 664 neuropathologically confirmed AD cases and 422 controls from the US. Initial analysis showed that APOE was the only signal to reach genome-wide significance after Bonferroni correction for multiple testing. In an effort to identify additional statistically significant data, the authors focused on 312,316 SNPs and divided the neuropathological sample into discovery (736 combined cases and controls) and replication (321 subjects) cohort (Reiman et al., 2007). This was augmented with an additional group of 364 AD cases and controls with clinical diagnoses. Additionally, stratification on APOE ε4 genotype was performed. The analysis revealed genome-wide significant association with five SNPs in the GAB2 gene. Follow-up studies have shown relative consistency regarding the role of GAB2 in AD pathophysiology. GRB2-associated binding protein 2 (GAB2) is expressed at particularly high levels in the prefrontal cortex and the hypothalamus. The growth factor receptor bound protein (GRB2), which binds GAB2, has been proposed to regulate signal transduction and is thought to influence phosphorylation of tau.

The study of Li et al. 2007

A subsequent study analyzed more than 400,000 SNPs from the Affymetrix 500K platform using 753 AD cases and 736 controls of northern European ancestry from

Canada (Li et al., 2007). Statistically most significant signals were followed up in the 418 AD cases and 249 control subjects. Once again, markers linked to APOE ε4 exhibited genome-wide association with statistical significance. In addition, four SNPs showed consistent evidence of association in both investigated sample groups, although none with genome-wide significance. Two of the aforementioned SNPs reside in the GOLM1 gene (also known as GOLPH2), which is involved in Golgi transmembrane trafficking. Together with a SNP located in an uncharacterized region on chromosome 9 (GWA_9p24.3), they showed association with risk of AD. The remaining identified SNP in an uncharacterized region on chromosome 15 (GWA_15q21.2) showed the strongest association with age of onset for AD.

The study of Abraham et al. 2008

Using a pooled DNA approach on 1082 AD cases and 1239 controls of Caucasian ancestry from UK, Abraham and coworkers tested the association of more than 560,000 Hap-Map-based SNPs on two Illumina platforms (Abraham et al., 2008). Additionally, in the follow-up stage of the study the authors used previously published "general disease" controls from the Wellcome Trust Case-Control Consortium as additional control subjects. In addition to five APOE-related SNPs, 109 nominally significant results in other loci have also been discovered. Among the nominally significant loci, SNP rs727153, residing in the haplotype block on chromosome 4q32 which includes the LRAT gene, showed the highest statistical significance. Due to the DNA polling approach and lack of independent case group in the follow-up stage, the results of the study still remain to be confirmed.

The study of Bertram et al. 2008

The first study to employ the family-based methods for the initial screening and replicant analyses utilized the Affymetrix 500K SNP platform to analyze 941 AD cases and 404 controls from 410 families of European descent (Bertram et al., 2008). Follow-up analyses were performed in 1767 AD cases compared to 838 controls from three independent collections consisting of 875 families, also of European descent. The authors used a novel family-based association approach that assesses disease status and age of onset jointly. Opting for a non-traditional approach to data analysis, the authors of the study first screened all of the markers to estimate the conditional power of each marker. In the second step, they computed statistical significance for family-based association corrected according to weights derived from the initial analysis. In concordance with other GWAS findings, the most significant association was observed with a marker in strong linkage disequilibrium (LD) with APOE ε4. In addition, four non APOE SNPs where detected, none of which were previously described as potential modifiers of AD risk or onset age. Three of these markers exhibited significant (GWA_14q31.2, CD33), or at least marginally significant (ATXN1) association in the follow-up experiments as well. It is important to note that GWA_14q31.2 also showed consistent replication in one of the two publicly available GWAS datasets.

The study of Beecham et al. 2009

One of the more recent GWAS studies performed on late onset AD patients involved 492 AD cases and 496 controls of Caucasian ancestry from the USA (Beecham et al., 2009). Promising signals were analyzed in a follow-up study involving 458 independent subjects. All of the samples were analyzed using the Illumina HapMap

550 platform Additional confirmation was performed by imputing the genotype of the previously published GWAS data (Reiman et al., 2007). Besides the APOE-related genetic markers, the second most significant signal was associated to SNP in the FAM113B gene on the chromosome 12q13. The aforementioned signal retained nominal significance after confirmation in the follow-up sample, but fell short of achieving significance in the imputed dataset. Additionally, it has not been validated in any subsequent association study. Four additional signals showed consistent evidence of association across both studies, namely DISC1, ZNF224, and two loci on chromosomes 4q28 and 6q14. The authors also undertook to reanalyze both GWAS datasets in an effort to assess the association of the genes included in the AlzGene database. The analysis revealed nominal evidence of association with a total of eight loci.

The study of Feulner et al. 2009

Using only a single-stage GWAS approach, Feulner and co-workers analyzed 491 AD cases and 479 younger controls from Germany, and focused on the top 10 genes included in the AlzGene database at the time of the study, as well as SORL1 (Feulner et al., 2009). Following the analysis on the Illumina HapMap550 microarrays, signals in 8 out of the 11 genes assayed revealed nominal significance of $P<0.05$ (GAB2, CHRNB2, CH25H, PGBD1, LMNA, PCK1, MAPT and SORL1). It is important to note that the study provided evidence of strong association at the extended APOE locus, particularly in the TOMM40 gene.

The study of Poduslo et al. 2009

The second study to utilize the family-based approach studied a total of nine affected and 10 unaffected individuals from France belonging to two large, multiplex AD pedigrees versus 60 unrelated controls from the Centre d'Etude du Polymorphisme Humain collection (Poduslo et al., 2009). Using the Affymetrix 500K SNP microarrays, the authors reported a single genome-wide significant association with 6 SNPs in the TRPC4AP gene. Interestingly, no association was reported for markers within or in LD with APOE. In an additional analysis, the authors identified a common 10-SMP haplotype in the TRPC4APwith increased frequency in the AD patients from the families compared with the control spouses. In the follow-up study on 199 AD cases and 85 controls, the same haplotype showed nominal significance. Transient receptor potential cation channel, subfamily C, member 4 associated protein (TRPC4A) has been implicated in regulation of the inflammatory cascade and calcium homeostasis. It is important to note that due to the small samples size, initial comparison of AD cases versus unrelated controls and inability to prove association with APOE-related SNPs, the results of the study require further independent confirmation.

The study of Carrasquillo et al. 2009

In one of the largest AD GWAS studies published to date, Carrasquillo and co-workers analyzed 844 late onset AD cases and 1255 controls from the US using the Illumina microarrays containing 300,000 HapMap-based SNPs (Carrasquillo et al., 2009). The only signals to exhibit genome-wide significance in this first stage of the study were located on chromosome 19 and showed strong LD with APOE ε4. In a follow-up study conducted using 1547 AD cases and 1209 controls, the authors

analyzed the top 25 SNPs, 10 of which were in LD with APOE. Using the combined dataset, the authors described one marker in the PCDH11X gene in the pseudoautosomal region of chromosome X. In concordance with this finding, the association was strongest in females, with OR estimates of 1.26 for heterozygotes and 1.75 for homozygotes, versus non-carriers of the putative risk allele. Male hemizygotes showed a similar trend, with OR of 1.18, although not at statistically significant level (P=0.07). Protocadherins are known to be involved in cell-cell adhesion and signaling, as well as neural development. Additionally, some protocadherins have been proposed as γ-secretase substrates, possibly competing with APP (Haas et al., 2005).

The study of Harold et al. 2009

Most recent studies published in the last two years have used either increased numbers of case and control subjects, or have employed novel analysis methods, such as assessing the CNV or association of specific quantitative traits and endophenotypes in AD patients.

Using 3941 late onset AD cases and 7848 controls from 13 different centers in Europe and US, Harold and coauthors analyzed association of more than 500,000 SNPs on Illumina microarrays (Harold et al., 2009). Besides APOE-related markers, rs11136000 in CLU and rs3851179 in PICALM exhibited genome-wide significance. Follow-up study of 2023 AD cases and 2340 controls confirmed nominal significance for the two non-APOE signals. Additional SNPs, such as CR1, showed P values of less than 10E-5, and among the 100 SNPs previously reported in GWAS studies to confer risk for AD, the authors were able to confirm PCDH11X and SORL1 at a P value of less than 0.05.

The studies of Lambert et al. 2009 and Lambert et al. 2010

Another large GWAS analyzed more than 500,000 SNPs in 2032 AD cases and 5328 controls from France using the Illumina Human 610-Quad microarray (Lambert et al., 2009). The first stage of the study revealed genome-wide significance of the rs11136000 marker in the CLU gene, in addition to the APOE-related markers. The significance of those SNPs was confirmed in the follow-up studies on 3978 AD cases and 3297 controls and in the combined series. In addition, a marker in the CR1 gene also achieved genome-wide significance in the combined series, while SNPs in PICALM and PCDH11X exhibited nominal significance. Clusterin (CLU) has been implicated in Aβ clearance from the brain and it has been proposed that it plays a role in Aβ fibrillogenesis and neurotoxicity (Holtzman, 2004). In a follow-up study, using the same dataset the authors utilized a different approach in analysis of the most significant associations by employing the two different gene set enrichment approaches and looking at the significantly associated groups of genes, rather than at individual genes (Lambert et al., 2010). The authors analyzed a large cohort of 2032 AD cases and 5328 controls of European ancestry using the Illumina Human 610-Quad Bead-Chips. After initial data filtering, the authors utilized the ALIGATOR software, developed to test over-representation of biological pathways in lists of significant SNPs from GWAS. They also performed the pathway based genome-wide association tests on SNP genotyping data with respect to the KEGG database. The results indicated 4776 SNPs nominally associated with risk of AD, which were assigned to 1395 genes involved in 173 KEGG pathways. Following the FDR correction, 5 physiological and disease-related KEGG pathways displayed significant over-representation of genes associated with the risk of AD. These included

pathways entitled AD, Regulation of autophagy, Natural killer cell mediated cytotoxicity, Antigen processing and presentation and RIG-I-like receptor signaling. The collective results of the study imply a strong role for and immune system dysfunction as a genetic risk for development of AD.

The study of Seshadri et al. 2010

In a recent effort to detect additional loci associated with AD, Seshadri and coworkers performed a 3-stage meta-analysis of new and previously published GWAS data on more than 35000 subjects of European ancestry, of which 8371 were AD cases (Seshadri et al., 2010). In stage 1 the authors performed a meta-analysis combining new genome-wide association data from Caucasian subjects which were part of the population-based Cohort for Heart and Aging Research in Genomics Epidemiology (CHARGE) consortium (Psaty et al., 2009) with GWAS data from the previous publications by Reiman and coworkers (2007) and Carrasquillo and coworkers (2009). The samples consisted of 8935 dementia-free individuals, of whom 973 developed incident AD over an average follow-up time of 8 years, and 2033 prevalent cases of AD who were compared to 14642 dementia-free controls. The analysis yielded 2078 SNPs with $P<10^{-3}$, which were subsequently studied in stage 2. After pooling the results for the aforementioned SNPs with the subjects from European AD Initiative (Lambert et al., 2009), in stage 2 of the study the authors were able to identify 38 SNPs corresponding to 10 loci with $P<10^{-5}$. The sample consisted of 2032 cases and 5328 controls. Finally, in stage 3 of the study, top SNPs from the 10 loci were meta-analyzed with the non-overlapping studies from Genetic and Environmental Risk in AD consortium (Harold et al., 2009) consisting of 3333 AD cases and 6995 controls. The final results in the Stage 3 of the study showed

genome-wide significance for 4 SNPs with $P<1.7x10^{-8}$ in addition to APOE-related markers. These included CLU and PICALM, as well as two novel loci on chromosome 2 and 19. The locus 2q14.3 is adjacent to the BIN1 gene, which is 1 of 2 amphiphysins and is expressed most abundantly in the brain and muscle (Wechsler-Reya et al., 1997). It has also been implicated in promotion of caspase-independent apoptosis and plays a role in neuronal membrane organization and vesicle trafficking (Wigge et al., 1997). The other locus 19q13.3 is adjacent to 6 genes, 2 of which are part of pathways linked to AD pathology. The genes in question are BLOC1S3, which has already been implicated in schizophrenia (Morris et al., 2008), and MARK4, which plays a role in neuronal differentiation (Moroni et al., 2006). The 4 associations were also replicated in an independent sample of Spanish origin, consisting of 1140 AD cases and 1209 controls (Antunez et al., 2009). The authors tried to assess whether APOE ε4, PICALM and CLU can improve predictive models for risk of incident AD in general population, but the findings showed that the predictive ability of the assayed genes was not clinically significant. The study represents an important milestone in AD research since it included the largest sample of clinic- and community-based cases and controls. However, the study showed limited power in detecting associations with small effect sizes and associations with rare variants.

The study of Naj et al. 2010

Expanding on the initial findings from the study published by Beecham and co-authors (Beecham et al., 2009), the authors conducted a follow-up GWAS by combining the initial 492 AD cases and 496 controls with an independent set of 439 AD cases and 608 controls of European ancestry (Naj et al., 2010). The new cohort

was analyzed in an effort to strengthen the power to identify novel genetic association signals. The most significant findings were replicated in an additional cohort containing 1338 cases and 2003 controls. As previously reported, APOE related SNP rs2075650 showed the most significant association with AD risk ($p=1.9x10^{-36}$). Among other significantly associated SNPs rs11754661 located in the MTHFD1L gene also showed significant association with AD ($p=4.7x10^{-8}$). The gene which encodes methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like protein is involved in tetrahydrofolate (THF) synthesis, which is an important step in homocysteine conversion to methionine. Elevated levels of homocysteine have been implicated in AD (Morris, 2003; Van Dam and Van Gool, 2010) and other neurodegenerative diseases (Herrmann and Knapp, 2002). It is important to note that association of MTHFD1L represents a novel finding for a genetic risk factor associated with AD, with viable biological and functional justification.

Copy number variation analysis in AD

The study of Heinzen et al. 2010

A growing number of studies have suggested that structural variation, including CNVs, may contribute to human disease (Estivill and Armengol, 2007) and a rare duplication of APP has already been linked to early onset AD. Using 331 AD cases and 368 controls of European ancestry, the authors conducted genome-wide genotyping on Illumina Human HapMap550K microarrays (Heinzen et al., 2010). GWAS results showed that only one SNP in the TOMM40 gene, located upstream of APOE ε4 gene, achieved genome-wide significance. The authors also evaluated previously reported genome-wide significant associations as well as 21 genes

implicated in candidate gene studies, but found no significant associations within their data set. The authors also preformed assessment of CNVs and, although they showed no clear excess of large deletions or multiplications, duplication within the schizophrenia and epilepsy associated risk region at 15q13.3 affecting the CHRNA7 gene was found in 2% of AD cases and 0.3% of control subjects. Additional analyses are required to fully assess the impact of rare structural variants in AD.

Quantitative-trait association studies in AD

The study of Potkin et al. 2009

Trying to apply quantitative trait association approach, Potkin and coworkers combined the neuroimaging data of hippocampal gray matter density MR measurements with results of the Illumina Human610 Quad microarray genotyping (Potkin et al., 2009). The study consisted of 172 AD cases and 209 controls of European ancestry from the Alzheimer's Disease Neuroimaging Inititative study. In the first part of the study, the authors performed a standard case-control study, which confirmed the association with APOE-related loci and also implicated TOMM40, a gene physically close to APOE, previously shown to exhibit significant association to AD. Application of the QT analysis identified additional 21 genes or chromosomal areas with at least one SNP with $P<10^{-6}$. The candidate genes included EFNA5, CAND1, MAGI2, ARSB and PRUNE2 genes, which have been implicated in ubiquination, oxidative necrosis, hippocampal development and dementia. The QT association studies offer a more objective measure compared to diagnostic categorization of case-control studies and can greatly increase statistical power of the findings. However, it should be emphasized that the authors analyzed only a

small number of mild AD patients, which are not fully representative of the disease. Additionally, selection of suitable quantitative traits in a complex disease as AD may prove to be a challenge.

The study of Stein et al 2010.

Using a similar approach, Stein and coauthors compared 3D profiles of temporal volume in MRI brain scans of AD patients, mildly impaired and healthy elderly subjects of European ancestry to genotyping data analyzed using the Human610-Quad microarrays (Stein et al., 2010). The study consisted of 173 AD cases, 361 MCI subjects and 208 control subjects. The results indicated two SNPs with genome-wide significance, namely rs10845840 ($P=1.260x10^{-7}$) located in chromosome 12 within an intron of the GRIN2B gene and rs 2456930 ($P=3.142x10^{-7}$), which lies in an intergenic region of chromosome 15. GRNB2 encodes the NR2B subunit of the NMDA receptor and is involved in learning and memory, structural plasticity of the brain, as well as neurodegeneration. Additional genes with nominal significance of $P<10^{-5}$ were also identified, and included RNF220, UTP20 and KIAA0743.

The study of Biffi et al. 2010

Another study utilizing the available genotyping and neuroimaging data from the Alzheimer's Disease Neuroimaging Initiative tried to assess the association of GWAS-validated and GWAS-promising novel AD loci on hippocampal volume, amygdale volume, white matter lesion volume (WML), entorhinal cortex thickness (ECT), parahippocampal gyrus thickness and temporal pole cortex thickness (TPT) (Biffi et al., 2010). The study included a total of 168 individuals with probable AD, 357 with MCI and 215 cognitively normal control individuals of European ancestry, who

have not been analyzed in previous association studies. The strongest association with clinical diagnosis was shown by APOE, while of the previously confirmed non-APOE AD loci, only CR1 was replicated in the data set with SNP rs1408077 showing a significant association (OR 1.27, FDR-corrected P=0.02). Novel AD associated variants included CNTN5 and BIN1. Using a genetic risk score analysis including 5 SNPs representing CLU, PICALM, CR1, CNTN5 and BIN1, the authors showed that predictive performance between score-based analysis and individual SNP analyses favored the cumulative effects model. As far as the correlation of genetic variants to the MR measurements is concerned, the results showed strong association of the APOE allele to all measures except WML. Further analyses showed association of GWAS-validated SNPs rs1408077 at CR1 with ECT and rs3851179 at PICALM with hippocampal coulume and ECT. Furthermore, novel associations were found for rs1501927 at CNTN5 with WML volume, parahippocampal gyrus thickness, TPT and ECT, as well as rs7561528 at BIN1 with TPT and ECT.

Discussion

Despite considerable advances in our understanding of genetic mechanisms involved in development of AD brought about by application of powerful high-throughput genome-wide association studies, full elucidation of the genetic disease risk factors is still evading scientists working in the field. In this review we have described results of eighteen studies utilizing different approaches to assess genome-wide association significance, varying from traditional case-control GWAS studies, to CNV analysis to quantitative-trait association studies. Collectively, the studies resulted in a large number of potentially interesting candidate genes. However, additional confirmation of the results is needed, not only through further association studies on larger samples of ethnically diverse subjects, but also on a functional level.

As is the case with many other genetically heterogeneous and complex diseases, the results show that the susceptibility genes confer relatively small increments in risk and a large number of common genetic variants may be required to account for the genetic risk in AD. However, the majority of the heritable component of the disease remains still largely unexplained. Therefore, to fully understand the genetic background of a given disease or trait, we must first understand the basis for this missing heritability (Gandhi and Wood, 2010). The missing heritability could be explained in several ways. Firstly, the majority of the genetic burden could be caused by independent factors of small effect size acting cumulatively to cause the disease. Secondly, the missing heritability might be explained by concerted action of multiple genes in an inter-dependent manner, through a, so far, unidentified pathway. Lastly, the burden might be the result of rarely occurring highly penetrant mutations.

This approach of searching for rare causal variants, described as having a minor allele frequency of less than 1%, each of modest effect, but residing within the same

functional unit, for example a gene (Morris and Zeggini, 2010), holds great promise. The aggregate role of low-frequency rare functional gene variants has not been properly evaluated in AD to date. These uncommon variants with relatively large effects might account for part of the unexplained heritability in AD. To establish the associations with rare variants it is necessary to perform direct mapping and rare variants within a sample must first be identified. The optimal way to identify rare variants is either through sequencing of candidate genes or large-scale sequencing of the entire genome. It is hypothesized that rare variants are more likely to be functional than common variants, which lead to an emerging interest in association studies of rare variants (Li and Leal, 2009). The completion of the 1,000 Genomes Project (Siva , 2008), which will perform whole genome re-sequencing of at least 1,000 genomes from 10 different ethnic backgrounds, and similar efforts are poised to shed much needed insight into the role of rare variants in pathophysiology of complex diseases.

Another crucial step in the fine-tuning of the initial GWAS results would include assessment of gene/gene and gene /environment interactions. These evaluations will present a substantial effort, since they require advanced computational methods and precise measurement of both the phenotype and the environmental factors. These efforts could help better understand the influence of environmental factors which could enhance disease onset, as well as disease progression (Manolio et al., 2009). Functional follow-up studies are a critical step in any observation stemming from an association study, conveying biological relevance to a possible genetic risk factor. Establishing the functional effect of a genetic factor is demanding, and includes numerous avenues of endeavor. These include employing similar approaches as in studying Mendelian genes, namely through generation of knock-in and knockout

animal models, and studying the effect of the loss-of-function or gain-of-function on the organism. Another approach may be to attempt to model the predicted genomic effect of any variant. However, these approaches are demanding, since the genomic effect of the causal variant is quite often unknown (Gandhi and Wood, 2010).

It is also important to note that most of the GWAS experiments have been conducted using individuals of European ancestry. Recent studies have indicated that genome-wide studies in a diverse set of population samples can offer improved power for discovery of causal genetic variants as compared to a study focusing exclusively on European population samples. The observed effect is dominated by the alleles found in lower frequency in populations of European-ancestry. The findings indicate that future GWAS and re-sequencing experiments could benefit greatly from inclusion of diverse population samples (Pulit et al., 2010).

One possible approach could be further study of CNVs across the genome in patients with AD. It is a well known fact that genomic structural variations are an important source of genetic variation and that they may increase the risk for a number of multifactorial diseases (Redon et al., 2006). Studies such as the one performed by Heinzen and coworkers (Heinzen et al., 2010) show promise in applying such approaches in the study of AD genetics. However, recent studies performed on large cohorts of patients with common complex diseases (Craddock et al., 2010), show that using common CNVs in association studies may not be as straightforward as previously imagined.

Finally, comparing GWAS to results of other omics approaches, such as expression profiling or proteomic analyses, may prove beneficial especially when predicting the genetic risk conveyed by the causal variants. Such prediction programs, aimed at determining susceptibility to AD, may prove helpful in the future as part of a

diagnostic algorithm or as a means of identifying high-risk individuals. Such approaches might provide an important step from association results to identification of biologically relevant causal variants and potential new diagnostic and therapeutic algorithms.

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Table 1. Summary of the results of genome-wide association studies in AD

Study	Type	Platform	Genes
Grupe et al., 2007.	GWAS case-control	Celera SNP platform	APOE, ACAN, BCR, CTSS, EBF3, FAM63A, GALP, GWA_14q32.13, GWA_7p15.2, LMNA, LOC651924, MYH13, PCK1, PGBD1, TNK1, TRAK1, UBD
Coon et al., 2007.	GWAS case-control	Affymetrix 500K	APOE
Reiman et al., 2007.	GWAS case-control	Affymetrix 500K	APOE, GAB2
Li et al., 2008.	GWAS case-control	Affymetrix 500K	APOE, GOLM1, GWA_9p24.3, GWA_15q21.2
Abraham et al., 2008.	GWAS case-control	Affymetrix 500K	APOE, LRAT
Bertram et al., 2008.	GWAS family-based	Affymetrix 500K	APOE, ATXN1, CD33, GWA_14q31.2
Beecham et al., 2009.	GWAS case-control	Illumina HapMap 550K	APOE, FAM113B
Feulner et al., 2009.	GWAS case-control	Illumina HapMap 550K	APOE/TOMM40
Poduslo et al., 2009.	GWAS case-control /family-based	Affymetrix 500K	TRPC4AP
Carasquillo et al., 2009.	GWAS case-control	Illumina HapMap 300K	APOE, PCDH11X
Harold et al., 2009.	GWAS case-control	Illumina HapMap 300/550K Illumina 610-quad	APOE, CLU, PICALM
Lambert et al., 2009.	GWAS case-control	Illumina 610-quad	APOE, CLU, CR1
Heinzen et al., 2009.	GWAS/CNV case-control	Illumina HapMap 550K	APOE/TOMM40, CHRNA7
Potkin et al., 2009.	GWAS/QT-AS case-control	Illumina 610-quad	APOE/TOMM40, EFNA5, CAND1, MAGI2, ARSB, PRUNE2
Naj et al., 2010.	GWAS case-control	Illumina 610-quad Illumine 1M	APOE/TOMM40, MTHFD1L, PVRL2, RDH13, GRIA4
Seshadri et al., 2010.	GWAS case-control	Affymetrix & Illumina various	APOE, CLU, PICALM, GWA_2q14.3, GWA_19q13.3
Stein et al., 2010.	QT-AS case-control	Illumina 610-quad	GRIN2B, GWA_15q22.2
Biffi et a., 2010.	QT-AS case-control	Illumina 610-quad	APOE,CR1, CNTN5, BIN1