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Genome-wide Association Study of Body Mass Index in Subjects with Alcohol Dependence

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Abstract

Outcomes related to disordered metabolism are common in alcohol dependence (AD). To investigate alterations in the regulation of body mass that occur in the context of AD, we performed a GWAS of BMI in African-Americans (AAs) and European-Americans (EAs) with AD. Subjects were recruited for genetic studies of alcohol or drug dependence, and evaluated using the Semi-Structured Assessment for Drug Dependence and Alcoholism. We investigated a total of 2,587 AAs and 2,959 EAs with DSM-IV AD diagnosis. In the stage-1 sample (N=4,137), we observed three genome-wide significant (GWS) SNP associations, rs200889048 ($p=8.98*10^{-12}$) and rs12490016 ($p=1.44*10^{-8}$) in EAs, and rs1630623 ($p=5.14*10^{-9}$) in AAs and EAs meta-analyzed. In the stage-2 sample (N=1,409), we replicated 278, 253, and 168 of the stage-1 suggestive loci ($p<5*10^{-4}$) in AAs, EAs, and AAs and EAs meta-analyzed, respectively. A meta-analysis of stage-1 and stage-2 samples (N=5,546) identified two additional GWS signals: rs28562191 in EAs ($p=4.46*10^{-8}$) and rs56950471 in AAs ($p=1.57*10^{-9}$). Three of the GWS loci identified (rs200889048, rs12490016, rs1630623) were not previously reported by GWAS of BMI

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Authors contributions

RP, HuZ, and JG were responsible for the study concept and design. HRK and JG were responsible for the recruitment of the samples. LAF was responsible for the genotyping and imputation. RP, AHS, HoZ and JG assisted with data analysis and interpretation of findings. RP drafted the manuscript. All authors provided critical revision of the manuscript for important intellectual content and approved final version for publication.

in the general population and two of them raise interesting hypotheses: rs12490016 – a regulatory variant located within *LINC00880*, where there are other GWAS-identified variants associated with birth size, adiposity in newborns, and bulimia symptoms which also interact with social stress in relation to birth size; rs1630623 – a regulatory variant related to *ALDH1A1*, a gene involved in alcohol metabolism and adipocyte plasticity. These loci offer molecular insights regarding the regulatory mechanisms of body mass in the context of AD.

Keywords

alcohol addiction; metabolic processes; GWAS; complex traits; ancestry; genetics

Introduction

GWAS of BMI in the general population indicated that this trait was significantly associated with genes involved in different substance dependence (SD)-susceptible mechanisms, such as neural function and energy balance (Hebebrand et al., 2010; Speliotes et al., 2010). Furthermore, dysregulated brain reward pathways may contribute to both SD and “food addiction” (Berthoud et al., 2011), suggesting partially shared pathogenic mechanisms of these traits.

Alcohol abuse is the third leading cause of preventable death in the United States (Mokdad et al., 2004), and alcohol dependence (AD) is experienced by ~14% of alcohol users (Grant et al., 2001). Several GWAS of AD have been performed, detecting different risk loci (Bierut et al., 2010; Edenberg et al., 2010; Gelernter et al., 2014; Quillen et al., 2014; Zuo et al., 2012). The most strongly supported risk locus in European- and African-ancestry populations is the ADH cluster, but other loci also play important roles in AD risk. No previous GWAS of BMI in subjects identified as AD has been performed, but a genome-wide gene-environment interaction analysis failed to find significant loci that interacted with alcohol consumption in relation to BMI (Velez Edwards et al., 2013). A recent study offered evidence in support of the hypothesis that there are six types of obesity and one of them is related to heavy alcohol drinkers (Green et al., 2015), suggesting that subjects with AD can have specific pathogenic mechanisms that affect body mass regulation.

Previous studies have focused on the effects of AD on BMI. AD subjects with a low level of alcohol drinking showed normal metabolic control, with alcohol intake being compensated by a decrease in non-alcoholic nutrients; conversely, AD subjects with high alcohol intake showed a loss of metabolic control, where alcohol accelerated metabolism and decreased fat mass and leptin levels (de Timary et al., 2012). Neurobiological investigation of AD subjects has indicated that BMI – independent of age, alcohol consumption, and common comorbidities – is correlated to regional concentrations of N-acetyl-aspartate (a marker of neuronal viability), choline-containing compounds (a marker of membrane turnover), creatine and phosphocreatine (markers of high energy metabolism), and myoinositol (a putative marker of astrocytes) (Gazdzinski et al., 2010). Genetic studies of BMI in AD subjects have all been candidate gene analyses and have yielded limited data. A longitudinal study of the effect of AD familial risk on BMI developmental changes observed significant

differences between males with high AD risk and those with low AD risk, and interaction of *DRD2* and *FTO* gene variation with risk status and sex (Lichenstein et al., 2014). Previous candidate gene studies of *FTO* in relation to AD reported nominally significant associations (Sobczyk-Kopciol et al., 2011; Wang et al., 2013), but the results are in some cases not concordant and no GWAS has confirmed these findings. Finally, exon sequencing analysis of the *POMC* gene, which encodes melanocortin peptides that are linked to SD and obesity risk, indicated that variation at this locus can contribute to risk for both traits (Wang et al., 2012). However, the effects of AD on BMI are complex and not well understood.

In the present study, we used GWAS to investigate the genetics of BMI in AD subjects, to identify AD-specific mechanisms. We analyzed data from a total of 5,546 subjects (stage-1 N=4,137; stage-2 N=1,409) with DSM-IV diagnosis of lifetime AD (2,587 AAs and 2,959 EAs), combining our samples with the Study of Addiction: Genetics and Environment (SAGE), which is available through dbGaP (accession number phs000092.v1.p) (Bierut et al., 2010).

Methods and Materials

Subjects and Diagnostic Procedures

Our stage-1 sample combined two independent populations of subjects with DSM-IV diagnosis of lifetime AD that were both genotyped on ~1M-SNP microarrays, our sample (Yale-Penn, N = 3,017) (Gelernter et al., 2014) and the SAGE sample (N = 1,120) (Bierut et al., 2010). A total of 1,981 AAs and 2,156 EAs were included. For the stage-2 analysis, we recruited an additional 606 AAs and 803 EAs with AD using the same criteria as the initial Yale-Penn cohort; these samples were genotyped on a sparser array.

The study was approved by the institutional review board at each site and we obtained written informed consent from each participant. Yale-Penn subjects were evaluated using the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) to derive DSM-IV diagnoses of lifetime AD and other major psychiatric traits (Pierucci-Lagha et al., 2005), and SAGE subjects were evaluated using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994). BMI was calculated on the basis of the height and weight that each participant reported during the SSADDA or SSAGA interview via the formula for BMI using inches and pounds. As performed also by previous GWAS of BMI (Monda et al., 2013; Speliotes et al., 2010), we used BMI values calculated on self-reported height and weight since the putative error in these data would likely bias the results toward the null outcome. Detailed information about the sample is available in our previous GWAS of alcohol dependence in AAs and EAs (Gelernter et al., 2014). Table 1 reports the characteristics of the analyzed populations.

Genotyping and Imputation

Yale-Penn samples were genotyped on the Illumina HumanOmni1-Quad v1.0 microarray containing 988,306 autosomal SNPs, at the Center for Inherited Disease Research (CIDR) or the Yale Center for Genome Analysis. Genotypes were called using GenomeStudio software V2011.1 and genotyping module V1.8.4 (Illumina, San Diego, CA, USA). The SAGE

samples were genotyped on the Illumina Human 1M array containing 1,069,796 total SNPs. The stage-2 cohort was genotyped using the Illumina HumanCoreExome array, which contains over 550,000 markers spilt between common and low-frequency variants. Principal component (PC) analysis was conducted in each sample (i.e., Yale-Penn, SAGE, and stage-2 cohort) using Eigensoft (Price et al., 2006) and SNPs that were common to the GWAS datasets and HapMap panel (after pruning the GWAS SNPs for linkage disequilibrium (LD) (r^2) > 80%) to characterize the underlying genetic architecture of the samples. Detailed information about pre-imputation quality control is available in our published AD GWAS (Gelernter et al., 2014). Imputation was performed using Impute2 software and the 1,000 Genomes reference panel. After imputation, we excluded SNPs with a minor allele frequency < 5% and poor imputation quality (Certainty < 0.9, Info < 0.3). Considering the SNPs common to GWAS cohorts (i.e., Yale-Penn and SAGE), 8,353,798 variants in AAs and 5,990,735 variants in EAs were included in association analyses.

Data analysis methods

Association tests were performed using the R package GWAF to fit a generalized estimating equations (GEE) model to correct for correlations among related individuals (Chen and Yang, 2010). GEE model analysis was performed considering pedigree information after checking genetic relatedness (i.e., confirming the relatedness of samples and excluding cryptic relatedness). We tested the association of the imputed minor allele dosage with BMI considered as the phenotype, and using DSM-IV cocaine dependence (CD) diagnosis, DSM-IV opioid dependence (OD) diagnosis, DSM-IV nicotine dependence (ND) diagnosis, sex, age, and the first three ancestry PCs, as covariates. Analyses were performed separately within each dataset and ancestry group, and the results were combined by meta-analysis using the program METAL (Willer et al., 2010). To prevent bias due to population stratification, we analyzed the AA and EA samples separately, and within each ancestry group we considered the first three principal components to adjust the genetic analysis. A P-value of $5*10^{-8}$ was the threshold for genome-wide significance (GWS) in the GWAS. Negligible inflation of P values was observed in both AAs and EAs (Supplemental Figures S1 and S2). To annotate the functional effects of the identified variants, we used information available in the UCSC genome browser (Kent et al., 2002), HaploReg (Ward and Kellis, 2012), Variant Effect Predictor (VEP) (McLaren et al., 2010), GTEx project (GTEx Consortium, 2013), and rSNPbase (Guo et al., 2014). Considering the results of GWAS in AAs and EAs, we performed a gene-based association analysis in each ancestry group using VEGAS software (Liu et al., 2010). Reference panels to correct for LD patterns in EAs and AAs were HapMap CEU and HapMap YRI, respectively. In gene-based association analysis, we estimated false discovery rate using the R package qvalue (Dabney and Storey, 2010), and considered q values < 0.05 as significant. Considering the gene-based association analysis data, we performed a protein-protein interaction (PPI)-based association analysis using the R package dmGWAS (Jia et al., 2011). Specifically, we defined PPIs of all genes with gene-based association using the Protein Interaction Network Analysis platform (PINA) v2.0 (Cowley et al., 2012), and subsequently we used R package dmGWAS to identify PPI modules enriched with small p values. We used both available independent population samples (AAs and EAs) to search for PPI modules enriched for BMI-associated genes (the “dual-evaluation” strategy). We applied a dense module search in the EAs and

follow-up analysis in AAs. The modules that remained significant after Bonferroni correction in AAs were considered to be relevant. Finally, we used DAVID 6.7 (Huang da et al., 2009) to perform functional annotation clustering, and generate a functional annotation chart using the results of the gene-based and PPI-based association analysis, respectively. High classification stringency and Bonferroni correction for multiple comparisons were considered in the DAVID analyses.

Results

Replication of loci previously associated with BMI in AAs and EAs

We evaluated whether previously identified BMI-associated loci could be replicated in our AA and EA GWAS cohorts (Supplemental Table 1 and Supplemental Table 2, respectively), considering the data provided by recent large GWAS of BMI in AAs and EAs (Monda et al., 2013; Speliotes et al., 2010). In AAs, the top reported BMI-associated variant, *SEC16B* rs543874, was replicated in our GWAS cohort (p = 0.027), as was another BMI-associated locus, *ADCY3* rs7586879 (p =0.021). We replicated *FTO* rs17817964 (p = 0.034) in our AA replication cohort. In EAs, the top-two BMI associated loci (*FTO* rs1558902 and *TMEM18* rs2867125) were both replicated in our GWAS cohort ($p = 7.0 * 10^{-6}$ and $p = 1.12*10^{-4}$, respectively), together with other BMI-associated loci (i.e., *ETV5* rs9816226, $p = 4.72*10^{-3}$; *NRXN3* rs10150332, p = 0.031; and *CADM2* rs13078807, $p = 7.48*10^{-3}$). *FTO* rs1558902 was also replicated (p = 0.018), and *RBJ* rs713586 (p = 0.039), *ETV5* rs9816226 (p = 0.021), *NRXN3* rs10150332 ($p = 4.04*10^{-3}$), and *NUDT3* rs206936 (p = 0.028) in our EA replication cohort.

Novel findings from SNP-based association analysis

Table 2a reports the top 20 variants in the SNP-based association analysis of BMI in EAs with AD. Among them, rs200889048 and rs12490016 were GWS in meta-analysis of EAs (rs200889048: EA meta-analysis $p = 8.98*10^{-12}$, Yale-Penn $p = 2.14*10^{-4}$, and SAGE $p = 2.52*10^{-10}$; rs12490016: EA meta-analysis $p = 1.44*10^{-8}$, Yale-Penn $p = 1.09*10^{-4}$, and SAGE $p = 2.16*10^{-5}$). Specifically, the minor alleles of rs200889048 and rs12490016 are both associated with increased BMI in AD subjects. Fourteen of the top 20 variants in EAs with AD are located in the *FTO* gene, the top BMI-associated locus for European ancestry reported in the largest previous meta-analysis (Speliotes et al., 2010). Table 2b reports the top 20 variants observed in the SNP-based association analysis of AAs with AD, none of which reached GWS in the Yale-Penn sample, SAGE sample, or meta-analysis of these two samples. Finally, to identify loci in which there was evidence for association in both populations, we performed a meta-analysis of AA and EA GWAS samples (Table 2c). One variant, rs1630623, was GWS in trans-population GWAS: the minor allele is associated with increased BMI in both AAs and EAs (trans-population $p = 5.14*10^{-9}$, EA $p = 1.85*10^{-7}$, and AA $p = 2.6610^{-3}$). We report regional Manhattan plots of the three GWS hits in supplemental Figure 3.

Functional annotation of GWS variants

Our GWAS of BMI in AD subjects identified three GWS variants: two in EAs (rs200889048 and rs12490016), and one in the combined-population analysis (irs1630623). The top variant

in EAs, rs200889048, is a 1-bp deletion located in an intergenic region. Considering the UCSF Brain DNA Methylation data (Maunakea et al., 2010) and information from HaploReg, we found that this variant is located in a methylated region, where different CpG sites are present, and affects 10 different regulatory motifs. The second GWS variant in EAs, rs12490016, is located in long noncoding RNA 880 (*LINC00880*). According to functional annotation by VEP and the information available from the UCSC Genome Browser, rs12490016 is located within a promoter flanking region (ENSR00001485403) near a CpG island (580 bp) and a K562 FAIRE peak (1,329 bp). rSNPbase classified rs12490016 as a regulatory SNP involved in distal regulation of several genes (*TIPARP*, *TIPARP-AS1, CCNL1, SSR3,* and *LINC881*). Additionally, recent GWAS identified the variants rs17451107, rs1482853, rs900400, and rs7624327 in the region of *LINC00880* as associated with birth weight, adiposity in newborns, and bulimia (Boraska et al., 2012; Horikoshi et al., 2013; Urbanek et al., 2013). The trans-population GWS variant, rs1630623, is located in *TMC1*, a gene associated with deafness and hearing loss (Kurima et al., 2002). It is 61 bp from an H3K27me3 region, and was classified by rSNPbase as a regulatory SNP involved in RNA binding protein mediated regulation. It is 175 kb downstream of *ALDH1A1*, a gene involved in alcohol metabolism, and in the regulation of the metabolic responses to a high-fat diet (Kiefer et al., 2012; Lind et al., 2012). Considering GTEx project data, we find that rs1630623 genotypes affect *ALDH1A1* gene expression significantly in whole blood ($p = 0.04$, $N = 168$)

Gene-based association analysis

In EAs, six genes (i.e., *KRTAP4-1*, *KRTAP4-3*, *KRTAP4-4*, *KRTAP4-5*, *KRTAP4-2*, *and KRTAP9-2*) showed significant associations with BMI in AD subjects ($q < 0.05$; Supplemental Table 3). However, these genes are clustered within 70 kb. Because the VEGAS software defines genes boundaries as ± 50 kb of the 5′ and 3′ UTRs, these observations are not independent. In AAs, gene-based association analysis did not reveal significant associations (Supplemental Table 4). To cluster genes on the basis of functional information, we used DAVID 6.7, considering nominally significant genes in AAs and EAs. After Bonferroni correction, there were three significant clusters observed in EAs, (Supplemental Table 5). However, two clusters are related to keratin and keratin-associated genes, located in a tight gene cluster on chromosome 17. Conversely, the top cluster is related to 36 Kruppel-associated (KRAB) proteins. Although some of these KRAB genes overlapped in the VEGAS analysis, 20 of them are completely independent. In contrast, no significant clusters were observed in AAs.

PPI-based association analysis

We used the results of GWAS in AAs and EAs to find PPI modules enriched for BMI-associated genes in AD. We used the dual evaluation approach of the R package dmGWAS, considering EAs as the discovery dataset and AAs as the evaluation dataset. In EAs, 12,125 PPI modules were identified. Considering the identified PPI modules in EAs, we then verified the enrichment of BMI-associated genes in AAs. One PPI module was significant after dual evaluation analysis (Figure 1). Eleven genes were included in this PPI network that was associated with BMI in AD. Performing a term enrichment analysis, we observed

several terms that remained significant after Bonferroni correction (Table 3). The top enriched terms are related to BMI-associated loci and cellular metabolism regulations.

Analysis of stage-1 findings in the stage-2 cohorts

We evaluated the stage-1 findings (with $p<5*10^{-4}$) in our independent stage-2 cohorts. Of these suggestive loci, 278, 253, and 168 of stage-1 suggestive loci replicated at nominal significance ($P<0.05$) and had effects that were directionally consistent with those in stage 1 (Supplemental Table 6, 7 and 8). A meta-analysis of stage-1 and stage-2 samples confirmed the GWS signal of rs200889048 in EAs ($p=9.44*10^{-10}$) and a suggestive GWS association of rs1630623 ($p = 9.73*10^{-8}$) in the AA-EA meta-analysis. Furthermore, this meta-analysis also identified two additional GWS variants: rs28562191 in EAs ($p=4.46*10^{-8}$) and rs56950471 in AAs ($p=1.57*10^{-9}$). Table 4 reports the relevant findings of the meta-analysis of stage-1 and stage-2 samples.

Discussion

Our GWAS of BMI in subjects with DSM-IV diagnosis of lifetime AD identified novel significant risk variants, genes, PPI networks, and pathways. Most of these significant findings appear to be specifically related to AD, since they were not reported in previous GWAS of BMI in the general population in considerably larger samples. Therefore, the predisposition to body mass changes in AD subjects could be partially related to AD-associated genetic mechanisms, providing specific evidence that alcohol intake can modify biological mechanisms and affect the genetic predisposition to this phenotypic trait. We analyzed EA and AA subjects and performed a trans-population investigation in a multiple-stage analysis to detect ancestry-specific and trans-population risk alleles (Polimanti et al., 2015). The stage-1 analysis (N=4,137) identified three GWS variants. In the stage-2 (N=1,409), we replicated numerous suggestive findings of stage-1 and observed concordant direction for two of the stage-1 GWS findings (rs200889048 and rs1630623), and a meta-analysis of stage-1 and stage-2 samples (N=5,546) identified two additional GWS loci. The loci highlighted by the meta-analysis of stage-1 and stage-2 samples were previously indicated by GWAS of BMI in general population: rs28562191 is located in the *FTO* gene, which is the top locus associated with BMI in populations with European ancestry (Speliotes et al., 2010), and rs56950471 is located in chromosome 11q23.3 where multiple GWAS identified variants associated with lipid traits and BMI in different population groups (Kiel et al., 2007; Ko et al., 2014; Shin et al., 2014). Conversely, the stage-1 GWS findings were not previously identified by GWAS of BMI in general population, indicating potential AD-specific loci associated with BMI.

In stage-1 EAs, we found two GWS associations. The top variant, rs200889048, was GWS in the SAGE cohort ($p = 2.53*10^{-10}$), a significant effect was observed in the Yale-Penn cohort ($p = 2.14*10^{-4}$), and the variant was highly significant in the meta-analysis of stage-1 cohorts ($p = 8.98*10^{-12}$). The meta-analysis of stage-1 and stage-2 cohorts also confirmed the GWS significant association of rs200889048 SNP with BMI in AD subjects ($p=9.44*10^{-10}$). This variant is located in a nongenic region, flanked by *CNTN3* (407 kb upstream) and *MIR444-1* (286 kb upstream) loci. Although the UCSF Brain DNA

Methylation data and HaploReg indicated that this variant is located in a highly methylated region and affects regulatory motifs, no further data seem to explain this genetic association. A number of databases are available for annotating gene function and regulation, but understanding the functional mechanism of GWAS-identified variants remains a key challenge.

The second GWS variant identified in stage-1 EAs, rs12490016, is located in *LINC00880*, flanked by the *LEKR* and *CCNL1* genes (EA meta-analysis $p = 1.44*10^{-8}$; SAGE $p = 2.16*10^{-5}$; Yale-Penn $p = 1.09*10^{-4}$). Previous GWAS have shown that other variants in *LINC00800* region were significantly associated with birth weight and adiposity in newborns and bulimia symptoms (Boraska et al., 2012; Horikoshi et al., 2013; Urbanek et al., 2013). Furthermore, a post-GWAS analysis indicated significant interplay between variants located in this region and social stress in relation to birth size (Ali Khan et al., 2012). Previous studies hypothesized that weight and weight gain during pre-natal life and infancy play a role in determining adulthood obesity (Bjerregaard et al., 2014). To address this issue, recent investigations analyzed the relationship between birth size, childhood and adulthood obesity, and behavioral factors. Genetic risk scores based on obesity studies in adults were significantly associated with postnatal growth, newborn adiposity, and "large for gestational age birth" phenotype (Chawla et al., 2014; Elks et al., 2014). Also highly relevant is a prospective analysis of the Helsinki Birth Cohort Study (N = 12,594) that showed pre- and post-natal growth to be associated with the risk for alcohol use disorders (AUD) later in life (Lahti et al., 2014). Finally, bulimia and AUD frequently co-occur, and some studies indicated that bulimia may share genetic factors with obesity and AD (Gamero-Villarroel et al., 2014; Muller et al., 2012; Trace et al., 2013). On the basis of these reported findings, the association of rs12490016 with BMI in AD provide further insight into the complex interplays between pre-natal, childhood and adulthood events in determining body mass changes.

The trans-population stage-1 analysis identified another GWS variant, rs1630623 (trans-population $p = 5.14*10^{-9}$; EA $p = 1.8510^{-7}$; AA $p = 2.6610^{-3}$). Although this variant is located in *TMC1*, a gene associated with deafness and hearing loss (Kurima et al., 2002), it is 175 kb downstream from *ALDH1A1* and its genotype is significantly associated with *ALDH1A1* gene expression. Beyond the association evidence and support for a functional effect of the associated SNP, *ALDH1A1* is an intriguing functional candidate as a BMI-associated gene in AD. It encodes aldehyde dehydrogenase family 1 member A1, an alcohol-metabolism enzyme, and it is expressed predominately in white adipose tissue (Kiefer et al., 2012). Although candidate gene studies have supported the association between *ALDH1A1* variants and alcohol use disorders (AUD) in different ancestry groups (Crawford et al., 2014; Lind et al., 2008; Liu et al., 2011), no GWAS of AUD showed this gene to be relevant; indeed our previous AD GWAS supported association in several alcohol dehydrogenase genes in both AAs and EAs, but not this particular locus. However, *ALDH1A1* variants were associated with blood alcohol concentration (Lind et al., 2012), confirming the role of this gene in alcohol metabolism. *ALDH1A1*, the protein product of which also catalyzes conversion of retinaldehyde to retinoic acid, is involved in different molecular processes, such as regulation of marrow adiposity, antioxidant defense, carcinogenesis, and neurodegeneration (Grunblatt and Riederer, 2014; Li et al., 2014;

Nallamshetty et al., 2014). Furthermore, recent animal experiments on Aldh1a1−/− mice demonstrated that the enzyme and its substrate retinaldehyde were involved in adipocyte plasticity and adaptive thermogenesis (Kiefer et al., 2012). These data are all consistent with the association of rs1630623 with BMI in AD, suggesting that *ALDH1A1* can play a relevant role in determining BMI in subjects with AD.

Our gene-based analysis in stage-1 EAs with AD identified six significant genes ($q < 0.05$). All these genes – *KRTAP4-1*, *KRTAP4-3*, *KRTAP4-4*, *KRTAP4-5*, *KRTAP4-2*, *and KRTAP9-2* - encode keratin-associated proteins, but because they are located in a tight gene cluster, the significant signals are not independent. However, although we cannot identify a specific source of the signal, the significant signal in the keratin-associated gene cluster appears to be reliable. Keratin and keratin-associated genes encode intermediate filament proteins, are expressed specifically in epithelial cells and their appendages, and are currently used as markers for various malignancies and other diseases (Upasani et al., 2004). One study highlighted a synergistic effect of alcohol consumption and BMI on serum concentrations of keratin-18 (Gonzalez-Quintela et al., 2011), a keratin marker of epithelial neoplasms. The authors suggested that this result probably reflects liver disease in obese subjects with risky alcohol drinking. The results of our gene-based analysis raise the possibility of a new scenario, in which keratin-related functions interact with alcohol drinking to influence BMI. However, further investigations are needed to elucidate the biological meaning of the association.

Functional annotation clustering analysis based on gene-based association identified three significant clusters. Two of these clusters are related to keratin and keratin-associated genes, and, for the reason discussed above, are due to non-independent results. In contrast, the top cluster is related to 36 KRAB genes, many of which are located on different chromosomes. The KRAB protein family includes 400 human zinc finger protein-based transcription factors (Margolin et al., 1994). Although KRAB proteins operate a well-defined transcriptional repression mechanism, there are few known biological roles or target genes of these proteins (Lupo et al., 2013). However, *in vivo* studies indicated that KRAB genes may be involved in obesity-related traits and metabolic homeostasis (Krebs et al., 2014; Scherneck et al., 2009). Furthermore, animal models indicated that alcohol consumption affected the gene expression regulation of zinc finger proteins (Curry-McCoy et al., 2013; Sun et al., 2014). On the basis of these findings, the significant functional annotation cluster related to KRAB genes may reflect underlying biology in which KRAB gene expression deregulation due to alcohol consumption in AD subjects is associated with metabolic changes that affect body mass.

Our PPI-based association analysis identified one significant module via the dual evaluation of stage-1 AA and EA samples. This module included 11 genes, eight of which were loci associated with BMI in AD ($p < 10^{-4}$). Enrichment analysis identified several significant terms related to genes involved in the PPI module significantly associated with BMI in AD subjects. The two most highly significant enriched terms were related to BMI-associated loci (i.e., *FTO* and *TMEM18*). The subsequent two significant terms were related to negative regulation in molecular processes, involving the genes *GPS1*, *UBC*, and *CDC20*. However, *GPS1* is the only gene associated with BMI in AD ($p = 3.14*10^{-4}$), whereas no significant

associations were present for *UBC* and *CDC20*. Two other significant terms are linked to keratin-related genes, which were non-independent in the gene-based analysis. The remaining significant terms were related to *UBC* and *CDC20*, which are linked to different ubiquitin-dependent processes. A consistent literature describes ubiquitin-dependent processes, and some evidence is also available about the role of these processes in adipocyte-related mechanisms (Dai et al., 2013; Kim et al., 2014; Nian et al., 2010). However, most of BMI-associated genes in the significant PPI module are not involved in the significant enriched terms, indicating no known pathways or mechanisms in this PPI module.

In conclusion, our GWAS of BMI in subjects with DSM-IV diagnosis of lifetime AD identified novel pathogenic mechanisms, indicating AD-specific genetic components of BMI. We believe that these help to elucidate a specific relationship between AD and BMI. The most intriguing findings suggested that i) AD could affect the genetic architecture of BMI via links between AD and intra-uterine growth and social stress during pregnancy; ii) there are interactions between alcohol metabolism and adipocyte plasticity in AD subjects; and iii) there is the potential involvement of keratin-associated and KRAB genes in the genetics of BMI in AD subjects. Although we obtained some provocative insights about genetic predisposition to BMI in AD subjects, larger study populations are needed to investigate this topic further. Taking all of the association evidence together, the present study demonstrates that GWAS can be useful in investigating the biological mechanisms related to the effects of AD on molecular processes. Because morbidity and mortality consequent to AD are also related to the adverse effects of alcohol use on a range of metabolic processes (including those that affect weight regulation), our results provide insights that may be useful in developing novel preventive and therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Gene	P value
KRTAP9-2	1.10E-05
KRTAP9-3	3.50E-05
FN3KRP	4.90E-05
TMEM18	5.60E-05
KRT33B	1.52E-04
GPS1	3.14E-04
FTO	3.26E-04
VANGL2	3.98E-04
CDC20	3.25E-01
UBC	5.03E-01
FAM91A1	8.14E-01

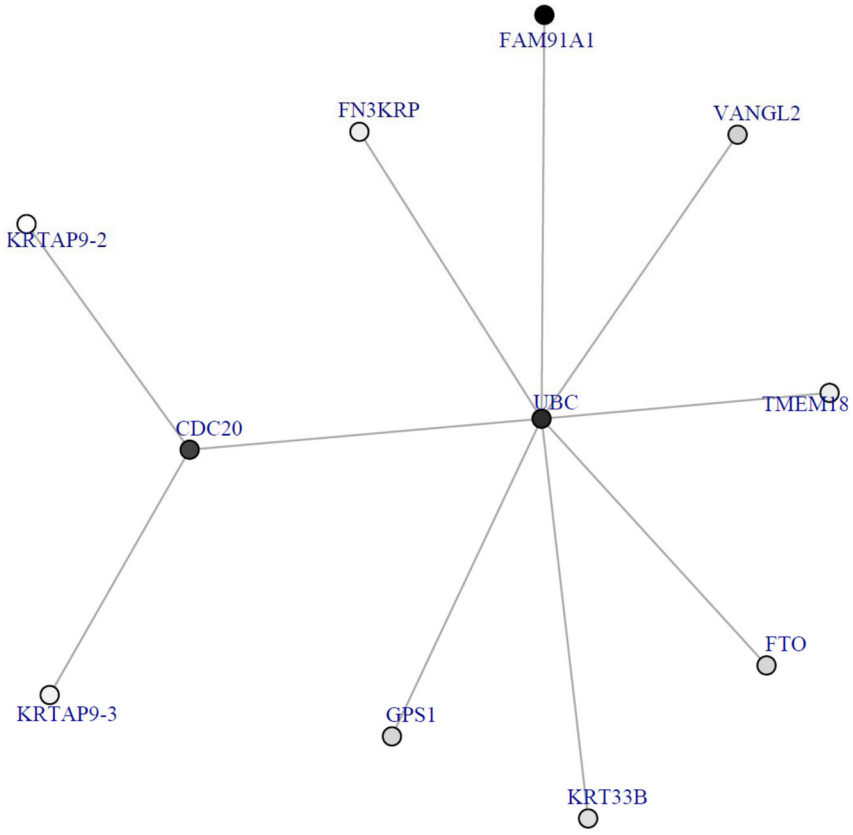

Figure 1.
PPI modules constructed with dual evaluation of stage-1 EAs and AAs. The grey color gradient of a node is proportional to its p values.

Table 1

Sample characteristics.

	Stage-1 cohort (N = 4,137)				Stage-2 cohort(N = 1,409)	
	Yale-Penn (N = 3,017)		SAGE (N = 1,120)			
	AA (N = 1,686)	EA (N = 1,331)	AA (N = 295)	EA (N = 825)	AA (N = 606)	EA (N = 803)
age-years, mean±SD	41.7±8.3	38.8±10.1	40.5±7.4	39.8±9.5	41.7±10.4	39.2±12.3
Female (%)	707 (42)	495 (37)	126 (42)	311 (38)	193 (32)	260 (32)
BMI < 19 (%)	23 (1)	26 (2)	3 (1)	17 (2)	10 (2)	13 (2)
BMI 19–24.9 (%)	447 (27)	439 (33)	68 (23)	307 (37)	140 (23)	280 (35)
BMI 25–29.9 (%)	590 (35)	494 (37)	119 (40)	316 (38)	220 (36)	303 (38)
BMI 30–34.9 (%)	339 (20)	246 (19)	56 (19)	121 (15)	127 (21)	131 (16)
BMI > 35 (%)	287 (17)	126 (9)	49 (17)	64 (8)	109 (18)	76 (9)

Table 2

Top-20 variants associated with BMI in AD considering EA subjects (**A**), AA subjects (**B**), and trans-population analysis (**C**), respectively. For EAs and trans-population association analysis, we reported also the p value of GIANT consortium (Stage 1). GWS p values are highlighted in bold. Minor allele frequencies (MAF) are calculated for each variant as meta-analysis of the investigated samples

Table 2a – EA association analysis

rsId	Chr	Location	MAF	Gene	P value (Yale-Penn)	P value (SAGE)	P value (meta-analysis)	Direction	P value (GIANT Stage 1)
rs6545444	2	55035355	0.068	EML6	4.13E-04	4.45E-04	7.53E-07	++	0.954
rs2116440	2	55037697	0.068	EML6	3.88E-04	4.34E-04	6.90E-07	++	0.99
rs200889048	3	74977426	0.063	NA	2.14E-04	**2.52E-10**	**8.98E-12**	++	NA
rs12490016	3	156838931	0.064	LINC00880	1.09E-04	2.16E-05	**1.44E-08**	++	NA
rs114337256	4	134164378	0.056	NA	2.33E-06	2.74E-02	3.88E-07	++	NA
rs1630623	9	75340239	0.177	TMC1	8.66E-04	2.70E-05	1.85E-07	++	NA
rs9937521	16	53799296	0.396	FTO	7.00E-06	6.01E-03	1.70E-07	−−	NA
rs28562191	16	53799303	0.390	FTO	8.44E-06	5.25E-03	1.73E-07	−−	NA
rs9937354	16	53799847	0.428	FTO	1.74E-05	9.88E-03	6.65E-07	−−	NA
rs9928094	16	53799905	0.428	FTO	1.74E-05	9.88E-03	6.65E-07	−−	1.35E-57
rs9930397	16	53799985	0.428	FTO	1.74E-05	9.88E-03	6.65E-07	−−	NA
rs9940278	16	53800200	0.428	FTO	1.74E-05	9.87E-03	6.65E-07	−−	NA
rs9939973	16	53800568	0.428	FTO	1.74E-05	9.85E-03	6.63E-07	−−	9.68E-58
rs9940646	16	53800629	0.428	FTO	1.74E-05	9.85E-03	6.63E-07	−−	1.43E-56
rs9940128	16	53800754	0.427	FTO	1.73E-05	9.84E-03	6.58E-07	−−	1.39E-57
rs1421086	16	53801343	0.428	FTO	1.73E-05	9.80E-03	6.57E-07	−−	NA
rs9923147	16	53801549	0.427	FTO	1.66E-05	9.75E-03	6.29E-07	−−	1.02E-57
rs1558901	16	53803187	0.429	FTO	1.43E-05	8.16E-03	4.52E-07	−−	NA
rs11075985	16	53805207	0.429	FTO	1.46E-05	7.70E-03	4.32E-07	−−	1.60E-57
rs1121980	16	53809247	0.429	FTO	1.36E-05	8.22E-03	4.34E-07	−−	1.78E-57

Table 2b – AA association analysis

rsID	Chr	Location	MAF	Gene	P value (Yale-Penn)	P value (SAGE)	P value (meta-analysis)	Direction
rs2046823	3	56779011	0.498	*ARHGEF3*	1.51E-06	2.08E-01	8.53E-07	++

Table 2b – AA association analysis

rsID	Chr	Location	MAF	Gene	P value (Yale-Penn)	P value (SAGE)	P value (meta-analysis)	Direction
rs2029466	3	56780003	0.438	*ARHGEF3*	1.00E-07	6.43E-01	3.52E-07	++
rs3772218	3	56782813	0.497	*ARHGEF3*	4.66E-06	1.20E-01	1.40E-06	++
rs35198830	3	1.74E+08	0.275	*NLGN1*	9.22E-06	1.27E-02	4.34E-07	++
rs1436526	4	86977277	0.055	*MAPK10*	1.03E-05	2.33E-02	7.66E-07	++
rs73834000	4	86981300	0.055	*MAPK10*	1.02E-05	2.33E-02	7.54E-07	++
rs1561154	4	86982434	0.055	*MAPK10*	1.01E-05	2.33E-02	7.51E-07	++
rs61454320	9	76530716	0.055	NA	5.51E-06	3.03E-02	4.94E-07	++
rs73470398	11	31849472	0.147	NA	1.21E-06	6.71E-02	2.16E-07	−−
rs16922496	11	31856488	0.147	NA	1.32E-06	7.08E-02	2.47E-07	−−
rs56950471	11	1.15E+08	0.262	NA	1.14E-05	7.32E-03	3.69E-07	++
rs74789538	15	36281417	0.086	NA	9.15E-07	1.89E-01	4.75E-07	++
rs79037607	15	36283130	0.088	NA	1.07E-06	1.50E-01	4.29E-07	++
rs113423262	15	36291751	0.086	NA	7.97E-07	1.53E-01	3.29E-07	++
rs116546602	15	36293032	0.086	NA	8.13E-07	1.53E-01	3.38E-07	++
rs1510391	15	36295848	0.086	NA	8.16E-07	1.61E-01	3.58E-07	++
rs1584033	18	3240255	0.100	NA	8.45E-07	4.14E-01	1.18E-06	++
rs1579766	18	3240288	0.099	NA	9.82E-07	4.13E-01	1.35E-06	++
rs205881	20	486771	0.336	*CSNK2A1*	5.60E-06	4.19E-02	6.56E-07	−−
rs34379659	22	39575873	0.089	NA	6.00E-07	3.60E-01	7.12E-07	++

Table 2c – trans-population association analysis

rsId	Chr	Location	MAF (AA/EA)	Gene	P value (trans-population meta-analysis)	Direction	P value (EA meta-analysis)	P value (AA meta-analysis)	P value (GIANT Stage 1)
rs2715093	7	50733034	0.493/0.483	*GRB10*	1.63E-07	−−−−	6.22E-05	6.96E-04	NA
rs2589626	9	75319272	0.300/0.165	*TMC1*	1.43E-07	++++	4.26E-06	5.00E-03	9.12E-01
rs1630623	9	75340239	0.284/0.177	*TMC1*	**5.14E-09**	++++	1.85E-07	2.66E-03	NA
rs1444825	9	75345502	0.283/0.166	*TMC1*	5.73E-08	++++	3.40E-06	2.73E-03	8.50E-01
rs1838486	9	75345816	0.281/0.166	*TMC1*	6.70E-08	++++	3.41E-06	3.11E-03	8.49E-01
rs2589608	9	75345891	0.276/0.166	*TMC1*	2.18E-07	++++	3.74E-06	7.68E-03	NA
rs2487465	9	75346270	0.281/0.166	*TMC1*	6.70E-08	++++	3.40E-06	3.11E-03	8.48E-01

Table 2c – trans-population association analysis

rsId	Chr	Location	MAF (AA/EA)	Gene	P value (trans-population meta-analysis)	Direction	P value (EA meta-analysis)	P value (AA meta-analysis)	P value (GIANT Stage 1)
rs2487466	9	75346354	0.276/0.166	*TMC1*	2.17E-07	++++	3.73E-06	7.69E-03	NA
rs1444826	9	75346619	0.281/0.166	*TMC1*	6.70E-08	++++	3.40E-06	3.12E-03	8.39E-01
rs1444827	9	75346847	0.291/0.162	*TMC1*	1.05E-07	++++	9.20E-06	2.24E-03	8.28E-01
rs2589610	9	75347643	0.282/0.167	*TMC1*	5.43E-08	++++	2.34E-06	3.38E-03	7.64E-01
rs1663738	9	75347852	0.281/0.166	*TMC1*	6.84E-08	++++	3.41E-06	3.16E-03	7.50E-01
rs10655647	9	75348082	0.276/0.166	*TMC1*	1.87E-07	++++	3.09E-06	7.70E-03	NA
rs2589632	9	75349586	0.283/0.166	*TMC1*	1.39E-07	++++	3.39E-06	5.70E-03	5.92E-01
rs2793168	9	75350343	0.282/0.166	*TMC1*	7.11E-08	++++	3.42E-06	3.26E-03	6.40E-01
rs2793169	9	75354006	0.284/0.166	*TMC1*	1.36E-07	++++	3.58E-06	5.41E-03	NA
rs2793170	9	75355918	0.282/0.166	*TMC1*	8.48E-08	++++	3.59E-06	3.66E-03	NA
rs2793171	9	75357642	0.282/0.166	*TMC1*	8.07E-08	++++	3.19E-06	3.80E-03	NA
rs2793172	9	75357660	0.291/0.166	*TMC1*	9.65E-08	++++	3.20E-06	4.40E-03	6.65E-01
rs1361531	9	75360357	0.282/0.166	*TMC1*	1.01E-07	++++	3.27E-06	4.48E-03	6.46E-01

Table 3

Term enrichment Analysis of PPI network associated with BMI in AD. Fisher exact test p values adjusted for Bonferroni correction are reported.

Term	Genes	Adjusted P value
OMIN:Six new loci associated with body mass index highlight a neuronal influence on body weight regulation	TMEM18, FTO	4.43E-04
OMIN:Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity	TMEM18, FTO	1.24E-03
GO:0043086~negative regulation of catalytic activity	GPS1, UBC, CDC20	4.43E-03
GO:0044092~negative regulation of molecular function	GPS1, UBC, CDC20	7.56E-03
GO:0005882~intermediate filament	KRTAP9-2, KRTAP9-3, KRT33B	8.10E-03
GO:0045111~intermediate filament cytoskeleton	KRTAP9-2, KRTAP9-3, KRT33B	8.64E-03
GO:0031145~anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	UBC, CDC20	1.24E-02
GO:0051436~negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	UBC, CDC20	1.24E-02
GO:0051352~negative regulation of ligase activity	UBC, CDC20	1.30E-02
GO:0051444~negative regulation of ubiquitin-protein ligase activity	UBC, CDC20	1.30E-02
GO:0051437~positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle	UBC, CDC20	1.35E-02
GO:0051443~positive regulation of ubiquitin-protein ligase activity	UBC, CDC20	1.40E-02
GO:0051439~regulation of ubiquitin-protein ligase activity during mitotic cell cycle	UBC, CDC20	1.46E-02
GO:0051351~positive regulation of ligase activity	UBC, CDC20	1.51E-02
GO:0031397~negative regulation of protein ubiquitination	UBC, CDC20	1.51E-02
GO:0051438~regulation of ubiquitin-protein ligase activity	UBC, CDC20	1.73E-02
GO:0051340~regulation of ligase activity	UBC, CDC20	1.89E-02
GO:0031398~positive regulation of protein ubiquitination	UBC, CDC20	2.05E-02
GO:0031396~regulation of protein ubiquitination	UBC, CDC20	2.86E-02
GO:0043161~proteasomal ubiquitin-dependent protein catabolic process	UBC, CDC20	2.97E-02
GO:0010498~proteasomal protein catabolic process	UBC, CDC20	2.97E-02
GO:0031400~negative regulation of protein modification process	UBC, CDC20	4.05E-02

Table 4

Meta-analysis of stage-1 and stage-2 samples of the GW significant variants in EAs, AAs and trans-population association analysis. GW significant results are highlighted in bold.

Ancestry	rsId	Stage-1		Stage-2 P value	Meta-analysis P value	Direction
		P value (Yale-Penn)	P-value (SAGE)			
EA	rs200889048	2.14E-04	**2.52E-10**	5.71E-01	**9.44E-10**	+++
	rs12490016	1.09E-04	2.16E-05	8.79E-01	1.94E-06	++−
	rs28562191	8.44E-06	5.25E-03	5.24E-02	**4.46E-08**	−−−
AA	rs56950471	1.13E-05	7.32E-03	1.03E-03	**1.57E-09**	−−−
AA-EA	rs1630623	Stage-1		Stage-2		Meta-analysis P value	Direction
		EA P value	AA P value	EA P value	AA P value		
		1.85E-07	2.66E-03	5.86E-01	8.12E-01	9.73E-08	++++++