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ANKRD7 and *CYTL1* are novel risk genes for alcohol drinking behavior

Xiang-ding CHEN,
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Osteoporosis research Center, Creighton University, Omaha, NE 68131, USA

Dong-hai XIONG,
Departments of Orthopedic Surgery and Basic Medical Sciences, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Tie-lin YANG,
Departments of Orthopedic Surgery and Basic Medical Sciences, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Yu-fang PEI,
Departments of Orthopedic Surgery and Basic Medical Sciences, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Yan-fang GUO,
Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Jian LI,
Departments of Orthopedic Surgery and Basic Medical Sciences, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Correspondence to: Dr. DENG Hong-wen, College of Life Sciences, Hunan Normal University, Changsha, Hunan 410081, China (Tel and Fax: 86-731-88872791. hwdeng@hunnu.edu.cn).

Institute contributions: Hunan Normal University: Research design, organization, manuscript drafting and recruitment of Chinese samples in Changsha; Xi'an Jiaotong University: Recruitment of Chinese samples in Xi'an; University of Missouri-Kansas City: Genotyping for Caucasian nuclear families samples, statistical analyses and critical revising of manuscript; Vanderbilt University Medical Center: Genotyping of initial Caucasian samples; University of Connecticut Health Center: Direction of alcohol dependence study and manuscript revising; Boston University School of Medicine: Genotyping for replication in unrelated Caucasians; Yale University School of Medicine: Replication research in unrelated Caucasians; Creighton University: Recruitment of initial unrelated Caucasian and nuclear family samples; University of Shanghai for Science and Technology: Genotyping of Chinese population.

Fang YANG,
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Feng PAN,
Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Li-jun TAN,
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Han YAN,
Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Xiao-gang LIU,
Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Shu-feng LEI,
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Departments of Orthopedic Surgery and Basic Medical Sciences, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Xi LI,
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Ling-ling NING,
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Xue-zhen ZHU,
Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Levy Shawn,
Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN 37232, USA

Kranzler Henry R.,
University of Connecticut Health Center, Farmington, CT 06030, USA

Farrer Lindsay A.,
Boston University School of Medicine, Boston, MA 02118, USA

Gelernter Joel,
Department of Psychiatry, Yale University School of Medicine, West Haven, CT 06516, USA

Recker Robert R., and
Osteoporosis research Center, Creighton University, Omaha, NE 68131, USA

Hong-wen DENG
Laboratory of Molecular and Statistical Genetics, Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Institute of Biology, Hunan Normal University, Changsha, Hunan 410081, China

Departments of Orthopedic Surgery and Basic Medical Sciences, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Key Laboratory of Biomedical Information Engineering of Ministry of Education and Institute of Molecular Genetics, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, Shannxi 710049, China

Systematic Biomedicine Research Center, University of Shanghai for Science and Technology, Shanghai 200093, China

Abstract

Background—Alcohol dependence (AD) is a complex disorder characterized by impaired control over drinking. It is determined by both genetic and environmental factors. The recent approach of genome-wide association study (GWAS) is a powerful tool for identifying complex disease-associated susceptibility alleles, however, a few GWASs have been conducted for AD, and their results are largely inconsistent. The present study aimed to screen the loci associated with alcohol-related phenotypes using GWAS technology.

Methods—A genome-wide association study with the behavior of regular alcohol drinking and alcohol consumption was performed to identify susceptibility genes associated with AD, using the Affymetrix 500K SNP array in an initial sample consisting of 904 unrelated Caucasian subjects. Then, the initial results in GWAS were replicated in three independent samples: 1972 Caucasians in 593 nuclear families, 761 unrelated Caucasian subjects, and 2955 unrelated Chinese Hans.

Results—Several genes were associated with the alcohol-related phenotypes at the genome-wide significance level, with the ankyrin repeat domain 7 gene (*ANKRD7*) showing the strongest statistical evidence for regular alcohol drinking and suggestive statistical evidence for alcohol consumption. In addition, certain haplotypes within the *ANKRD7* and cytokine-like1 (*CYTL1*) genes were significantly associated with regular drinking behavior, such as one *ANKRD7* block composed of the SNPs rs6466686-rs4295599-rs12531086 ($P = 6.51\times10^{-8}$). The association of alcohol consumption was successfully replicated with rs4295599 in *ANKRD7* gene in independent Caucasian nuclear families and independent unrelated Chinese Hans, and with rs16836497 in *CYTL1* gene in independent unrelated Caucasians. Meta-analyses based on both the GWAS and

replication samples further supported the observed significant associations between the *ANKRD7* or *CYTL1* gene and alcohol consumption.

Conclusion—The evidence suggests that *ANKRD7* and *CYTL1* genes may play an important role in the variance in AD risk.

Keywords

alcohol dependence; ANKRD7; CYTL1; genome-wide association study

Alcohol dependence (AD) is a complex behavioral disorder characterized by impaired control over drinking and a syndrome of serious problems related to alcohol abuse.[1] It is determined by both genetic and environmental factors with an estimated heritability of 40%–60%.[2] Although the prevalence of AD is moderate,[3] it is not easy to choose the best AD phenotype for genetic research because of the difficulties in psychiatric diagnosis of AD through personal interviews.[4] A number of alcohol-related phenotypes have been proposed and used in AD genetic studies,[5] such as broader definition of AD, withdrawal symptoms after cessation of heavy alcohol intake, history of alcohol-induced blackouts, age of onset of regular drinking, age at first drunkenness, alcohol drinking, maximum number of drinks,[6] daily alcohol consumption,[7] heavy consumption[8] and tolerance to alcohol's effects.[9]

Recent genetic research on AD employing both genome-wide linkage scans (GWLS)[6,8,10-18] and genome-wide association studies (GWAS)[19-23] have provided preliminary data of the genetic determination of AD. For example, GWLS results have implicated the importance of the genomic region-4q to AD risk;[6,8,10-13] and a GWAS using 100K single-nucleotide polymorphisms (SNPs) microarray in pooled DNA samples found 188 SNPs that were potentially associated with AD.[20] In particular, the state-of-the-art GWAS is a powerful tool for identifying susceptibility alleles of complex diseases.[24] However, currently a few GWAS or high-throughput association studies have been conducted for AD,[19,20,22,23] leaving the majority of the genetic factors underlying AD remain unknown.

In this study, we conducted a GWAS in a sample of 904 unrelated Caucasian subjects using Affymetrix 500K SNP microarrays, for one commonly used AD phenotype- alcohol drinking. Alcohol drinking information is obtained by self-reporting of the subjects. In Diagnostic Interview for Genetic Studies (DIGS),[25] question 5 in section of ALCOHOL ABUSE AND DEPENDENCE is “Did you ever drink regularly—that is, at least once a week, for six months or more?” (*http://zork.wustl.edu/nimh/home/m_DIGS_2.0.html/*).

Obviously, alcohol drinking is a necessary criterion for diagnosis of AD. For example, if a person does not drink any alcohol every week, he/she certainly does not have AD problems. Alcohol drinking is genetically determined because of its high heritability of 40% as calculated by our study of 6006 Caucasians from 1131 pedigrees (data not shown). It was also reported that alcohol drinking behavior and AD may have common genetic causes.[26] The present GWAS results suggested the importance of the ankyrin repeat domain 7 (*ANKRD7*) and cytokine-like1 (*CYTL1*) genes in the determination of alcohol drinking; and this implicated their potential importance for AD risk.

METHODS

Subjects

Nine hundred and four unrelated Caucasian subjects were recruited from Omaha and its surrounding areas in Nebraska for initial GWAS. Only healthy people (defined by the exclusion criteria that are detailed below) were included in the analysis. The sample of 904 subjects for this alcohol drinking study was part of our initial GWAS sample comprising 1000 unrelated Caucasians for osteoporosis studies,[27,28] of which 96 subjects were excluded because we do not have the alcohol drinking or age information for them. The other exclusion criteria were: subjects with certain conditions were excluded, including chronic disorders involving vital organs (heart, lung, liver, kidney, brain), serious metabolic diseases (diabetes, hypo- and hyper-parathyroidism, hyperthyroidism, etc.), skeletal diseases (Paget disease, osteogenesis imperfecta, rheumatoid arthritis, etc.), chronic use of drugs affecting bone metabolism (hormone replacement therapy, corticosteroid therapy, anti-convulsant drugs), malnutrition conditions (such as chronic diarrhea, chronic ulcerative colitis, etc.), and so forth. The study was approved by the Institutional Review Board at Creighton University. Informed-consent documents were obtained from all study participants before they entered the study.

Phenotypes

In this study, “alcohol drinking” indicates whether a study subject reported regularly drinking at least once every week and has two values: 1 for yes and 0 for no. “Alcohol Consumption” is graded as 0, 1, 2, 3 and 4, representing the number of alcohol drinking as 0, 1–2, 3–6, 7–10 and >10 times per week, respectively. Regular alcohol drinking in early ages was associated with later AD.[29] Relevant characteristics of the study subjects were summarized in Table 1. The mean percentages of drinkers were 83.2% for males and 74.0% for females.

Genotyping

Genomic DNA was extracted from human peripheral blood using a commercial isolation kit (Gentra systems, Minneapolis, MN, USA). Genotyping with the Affymetrix Mapping 250K Nsp1 and Affymetrix Mapping 250K Sty1 arrays was performed at the Vanderbilt Microarray Shared Resource at Vanderbilt University Medical Center, using the protocol recommended by the manufacturer. Briefly, genomic

DNA was first digested with either Nsp1 or Sty1 and then ligated to adapters. One primer amplification was performed to amplify the ligation products in a polymerase chain reaction (PCR). The amplified DNA was purified and fragmented. Samples with fragment distributions were hybridized to the Affymetrix array. Arrays were stained, washed and scanned with the Affymetrix array scanner 30007G. Data management and analyses were performed using the Affymetrix GeneChip Operating System. Genotyping calls were determined from the fluorescent intensities using the DM algorithm with a 0.33 confidence score setting and the BRLMM algorithm. According to Affymetrix guidelines, DM calls were used for quality control. Specifically, subjects with DM call rates <93% were subject to re-genotyping. All 904 subjects passed this quality control criterion. The BRLMM

algorithm was used for the association analyses due to its improved genotype calling. BRLMM clustering was performed with 94 samples per cluster. The final average BRLMM call rate across the entire sample was 99.14%. However, of the initial full-set of 500 568 SNPs, we discarded 32 961 SNPs whose sample call rates were less than 95%, 36 965 SNPs with allele frequencies deviating from Hardy-Weinberg equilibrium (HWE) (P <0.001) and 51 323 SNPs with minor allele frequencies (MAFs) < 1%. Consequently, association analyses were performed using the final SNP set containing 379 319 SNPs.

Replication studies

To validate our GWAS results, we performed replication studies using three independent samples: (1) 1972 Caucasians in 593 nuclear families; (2) 761 unrelated Caucasian subjects; (3) 2955 unrelated Chinese. The selection criteria for replication include statistical significance, haplotype block structure and minor allele frequency. Specifically, ten SNPs of *ANKRD7* and one SNP of *CYTL1* showed most significant associations in initial GWAS. Together with the linkage disequilibrium (LD) situations of *ANKRD7* SNPs, we chose four SNPs in *ANKRD7* and one SNP in *CYTL1* for the replication analyses.

Caucasian nuclear Families—A total of 1972 Caucasian subjects from 593 nuclear families living in Omaha, Nebraska were included in the analyses. The sample is composed of 831 parents (age range: 38.3–79.7 years), 246 male children (age range: 19.6–59.2 years) and 895 female children (age range: 19.0–61.1 years). The 40.3%, 35.9%, 18.5% and 5.3% of these nuclear families have 1, 2, 3 and more than 3 children, respectively, resulting in 824 sibling pairs and 1470 parent-offspring pairs. The overall alcohol drinking rates were 75.8% for females (age: (47.8±15.2) years) and 83.5% for males (age: (52.8±16.2) years), respectively. The genotyping concordance rate for duplicate samples was 99.7% through competitive allele specific PCR.[30] Association tests were carried out by the use of family-based association test (FBAT) software.

Unrelated Caucasian subjects—A total of 761 unrelated Caucasian subjects previously explored for genetic determination of nicotine dependence,[31] were used as the second replication sample. The mean age of these subjects at recruitment were (37.8±10.2) years, and the AD rate was 42.5%. The alcohol-related phenotype-maximum drinking was used for replication. Maximum number of drinks as an alcoholism phenotype has been used in the genetic dissection of AD.[6] All of the SNPs were genotyped using TaqMan assay and FBAT was conducted to test for association.

Chinese population—A total of 2955 healthy, unrelated Chinese Han adults from Xi'an and Changsha cities in China were used as the third replication sample. The study was approved by the Institutional Review Boards of Xi'an Jiaotong University and Hunan Normal University, respectively, and all study participants provided written informed consent before being enrolled into the study. The case sample of alcohol drinking comprised 536 subjects: including 431 males aged (29.6±10.6) years and 105 females aged (32.3±14.2) years. The control sample consisted of 2419 subjects, including 1006 males aged (30.9±13.3) years and 1413 females aged (35.7±15.8) years. The mean percentages of drinkers were 30.0% for males and 7.0% for females. Exclusion and inclusion criteria were

the same as those adopted in the initial Caucasian GWAS sample. Genotyping was performed using the MassARRAY system with MALDI-TOF mass spectrometry (Sequenom, Inc., San Diego, CA, USA).[32] The concordance rate for duplicate samples was 99.8%. Allelic and genotypic association tests were conducted using HelixTree.

Statistical analysis

Allelic, genotypic and haplotypic association tests were performed using HelixTree 5.3.1 (Golden Helix, Bozeman, MT, USA). The haplotype trend regression (HTR) approach was used to test haplotype associations.[33] The GWAS level significance threshold was set at 4.2×10^{-7} based on the criterion adopted by the previous GWAS researches.[34] False discovery rate (FDR) was also applied to address the multiple testing problems. Haploview (*http://www.broad.mit.edu/mpg/haploview/*) was used to analyze and plot LD patterns.[35] MAPPER (*http://mapper.chip.org*) was used to search for transcript factor binding sites.[36] In addition, EIGENSTRAT was used to minimize spurious associations due to population stratification,[37] though no evidence of population stratification was found.

The *z* transformation method was applied to combine *P* values from different samples for meta-analysis.[38] Each *P* value is transformed into a z score, a normal deviate via the normal probability transformation. The sum $s=\sum_{i=1}^{n} z_i$ has a normal distribution with N(0, *n*), thus, $s/\sqrt{n}$ has a normal N(0, 1) distribution. The combined *P* value is computed as the normal distribution function associated with $s/\sqrt{n}$.

RESULTS

Initial GWAS results

In the initial GWAS, ten SNPs in the *ANKRD7* gene were most significantly associated with alcohol drinking (FDR *q* values <0.05). These SNPs still showed highly suggestive associations with alcohol drinking adjusted by the significant covariates of sex and age (Table 2). Consistent results were obtained in HTR (haplotype trend regression) analyses for the 5-SNP haplotype windows consisting of *ANKRD7* SNPs rs7812233-rs4727889-rs6466686-rs4295599-rs12531086 ($P=2.47\times10^{-7}$, FDR $q=0.02$) and rs4320489-rs12534529-rs10230384- rs10271557-rs11487118 ($P=4.76\times10^{-7}$, FDR $q=0.02$) (Figure 1). In addition, the haplotype block *rs6466686-rs4295599-rs12531086* within *ANKRD7* (Block 9 in Figure 1) was highly significantly associated with alcohol drinking ($P=6.51\times10^{-8}$).

Possible functional significance of the most promising SNPs in *ANKRD7* was predicted through the analysis of the transcript factor binding sites. The results suggested that the change of alleles at certain SNP loci can alter the binding affinity of some transcriptional factors such as REVERB-alpha and SRY (Table 3). This may lead to the altered expression of *ANKRD7* gene that could be associated with the variance in alcohol drinking behavior.

For *CYTL1*, the 5-SNP haplotype window consisting of rs16836480-rs11728854-rs16836497-rs11732156- rs11723786 showed the most significant association with alcohol drinking ($P=4.42\times10^{-7}$, FDR $q=0.02$) (Figure 2). In allelic and genotypic association tests, *rs16836497* in *CYTL1* demonstrated significant associations with alcohol drinking (*P* =

1.08E-06 and 3.36E-06, respectively). In our genotyped SNP set, *rs16836497* was an independent SNP that was not in strong linkage disequilibrium (LD) with other *CYTL1* SNPs (Figure 2).

Replication results

Replication studies were conducted in the three independent samples. The threshold for judging significant replications was set at $P \leq 0.05$. Interestingly, the *ANKRD7* SNP *rs4295599* and the *CYTL1* SNP *rs16836497* showed the most significant associations with alcohol consumption in the replication samples. The combined *P* values from meta-analyses reached 5.79×10^{-5} and 7.46×10^{-5} for those two SNPs, respectively (Table 4). However, replication studies did not reveal the significance for alcohol drinking trait. Figure 3 shows the alcohol drinking rates stratified by the genotypes of the replicated SNPs in the *ANKRD7* and *CYTL1* genes, which showed significant differences ($P < 0.05$).

DISCUSSION

GWAS is beginning to reveal genes contributing to the risk of AD.[19-23] In our GWAS using Affymetrix 500K SNP chips, we identified two genes – *ANKRD7* and*CYTL1* that were significantly associated with an important phenotype for AD: alcohol drinking behavior, which was reported to share the common genetic components with AD.[26] In particular, the significant associations of *ANKRD7* and *CYTL1* genes with alcohol drinking were successfully replicated in the other independent Caucasian and Chinese samples for alcohol consumption, suggesting the validity of our initial GWAS findings. It should be noticed that Caucasians have much higher alcohol drinking rate than Chinese, partially due to the different polymorphisms of the alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) genes in different races.

ANKRD7 gene is located on chromosome 7q31. In the Collaborative Study on the Genetics of Alcoholism (COGA) sample, the 7q31 region containing *ANKRD7* was significantly linked to AD and the other AD related phenotypes.[39] In another genome-wide scan from the Framingham Heart Study (FHS), sib-pair regression analysis provided nominal evidence for linkage of alcohol consumption to the regions of 7q21, 7q32 and 7q36 (*D7S2204*, $P=0.0027$; *D7S1804*, $P=0.0049$; and *D7S559*, $P=0.001$, respectively)[40] that are very close to 7q31. These lend support to our detected significant associations between *ANKRD7* and alcohol drinking.

Functional studies of *ANKRD7* provide additional evidence supporting its importance to AD. First, *ANKRD7* was differentially expressed in cerebella between the inbred long-sleep (ILS) mice and the inbred short-sleep (ISS) mice.[41] In general, ILS mice are more sensitive to ethanol than ISS mice, and the expression level of *ANKRD7* in the cerebella of ISS mice was much higher than that of ILS mice ($P \leq 0.05$) (*http://www.ncbi.nlm.nih.gov/projects/geo/*). Because of the high conservation in the sequence of the *ANKRD7* gene between humans and mice, the differential expression of *ANKRD7* in mice with discordant alcohol-related traits may also support the importance of this gene to alcohol-related traits in humans. Furthermore, it was reported that the *ANKK1* (ankyrin repeat and kinase domain containing 1) gene was significantly associated with AD.[42,43] Since ANKK1 protein is

similar to ANKRD7 protein in structure (*http://www.ncbi.nlm.nih.gov/blast/Blast.cgi/*), it was logical to deduce that *ANKRD7* was involved in the etiology of AD in a similar way.

We also explored the potential functional changes that may be caused by different alleles of *ANKRD7* SNPs (Table 3). For example, one REVERB-alpha binding site around *rs7812233* in *ANKRD7* will be removed for the"A" allele. REVERB-alpha is encoded by nuclear receptor subfamily 1 group D member 1 (*NR1D1*) gene, which is related to the function of vitamin D3, thyroid and sex hormones that is important to alcohol metabolism. The deletion of the binding site of REVERB-alpha may affect the expression of the *ANKRD7* gene at the transcriptional level, which could result in the variance in alcohol drinking and AD risk.

CYTL1 in 4p16 is a cartilage-related gene.[44] Both our initial GWAS and the follow-up replication results supported the association of *CYTL1* with alcohol consumption. Previously, it was also reported that 4p15.3 was linked to the maximum alcohol consumption.[40] The linkage of 4p16 to psychiatric disorders has also been reported, such as its significant linkages to bipolar disorder (BPD)[45] and schizophrenia.[46] Since AD is correlated with the psychiatric traits,[47] the above linkages indirectly support the existence of some genetic factors in 4p16 that may contribute to AD phenotypes. This is consistent with our data showing that *CYTL1* should be among the list of AD candidate genes in the 4p16 region.

In summary, our GWAS and replication results provide the evidences supporting that *ANKRD7* and *CYTL1* are significantly associated with alcohol drinking behavior, which is an important phenotype of AD. Replication by other investigators and the elucidation of the underlying molecular mechanisms are needed to verify our findings.

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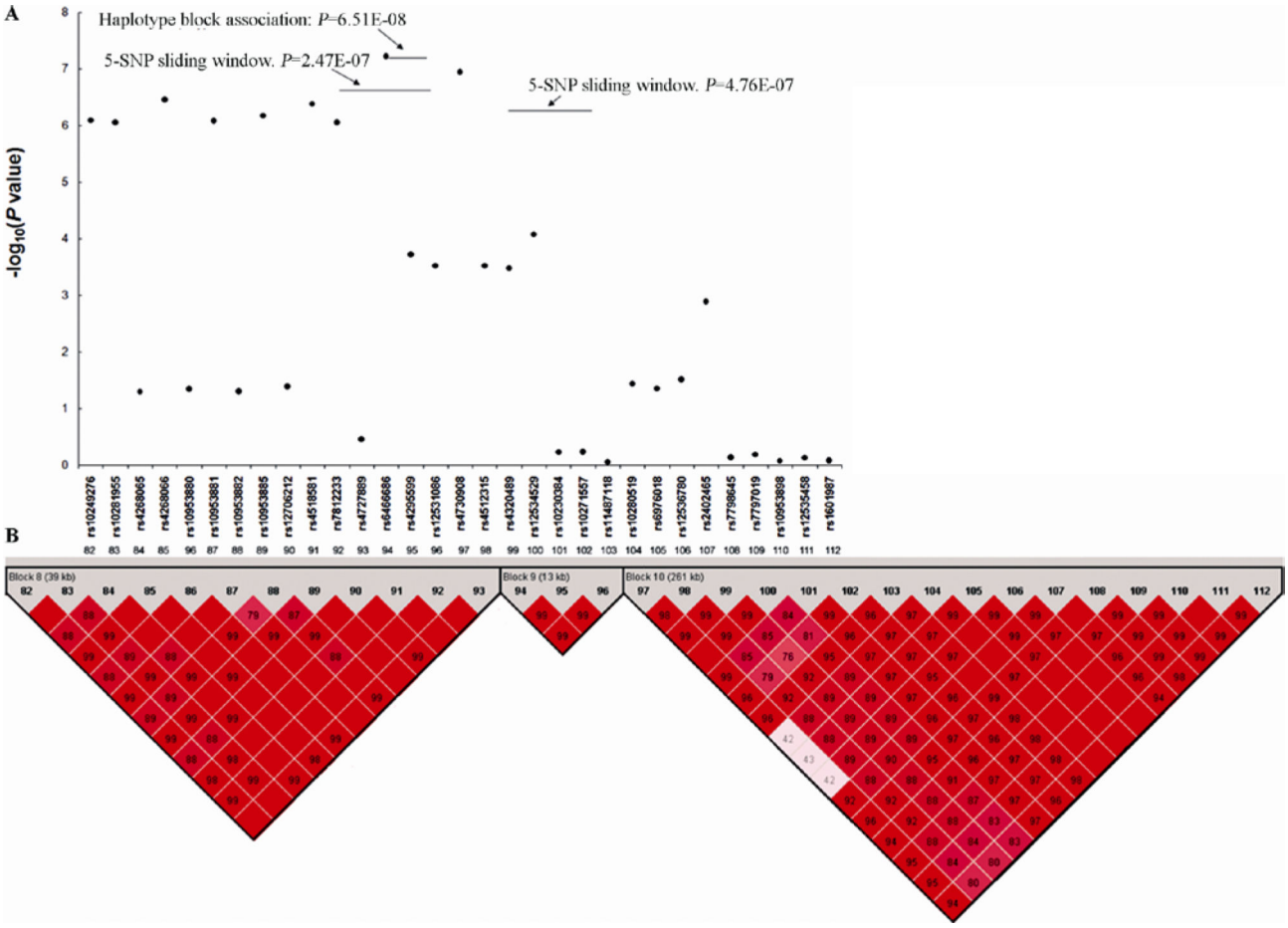

Figure 1.

Associations of *ANKRD7* gene with alcohol drinking in the total initial Caucasian sample. **A:** Assocoation results. The *y*-axis showed the -$\log_{10}$ *P* values and *x*-axis showed the identity of 31 SNPs in three haplotype blocks which contained significant SNP association with alcohol drinking. Significant haplotypes were plotted with the solid lines. **B:** Linkage disequilibrium pattern of *ANKRD7* gene in the three haplotype blocks. The linkage disequilibrium extent was denoted using the pairwise r^2 values.

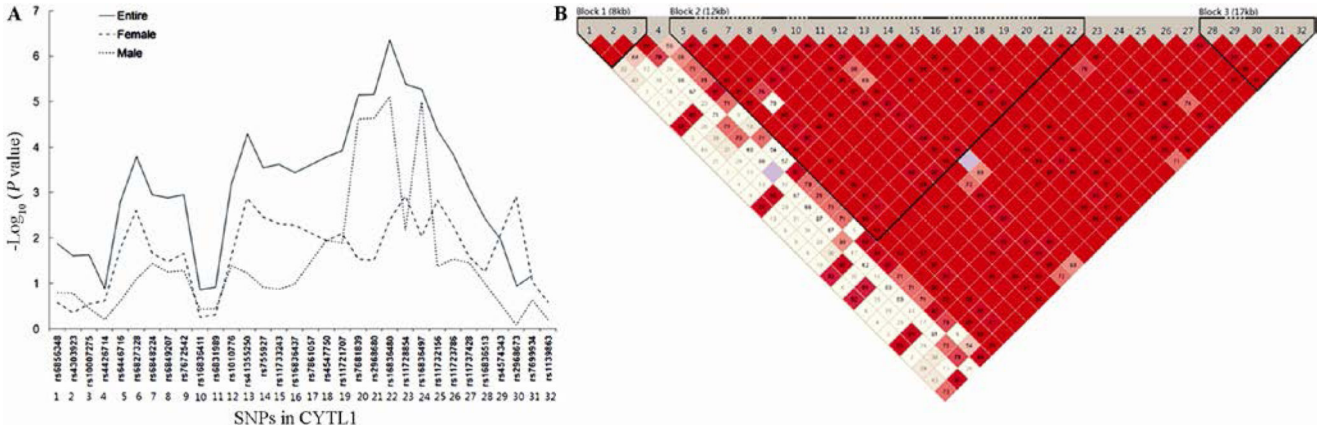

Figure 2.

Associations of *CYTL1* with alcohol drinking in the initial Caucasian sample. **A:** Assocoation results. The γ-axis showed the -$\log_{10}$ *P* values and χ-axis showed the identity of SNPs. **B:** Linkage disequilibrium pattern of the *CYTL1* gene. The linkage disequilibrium extent was denoted using the pairwise r^2 values.

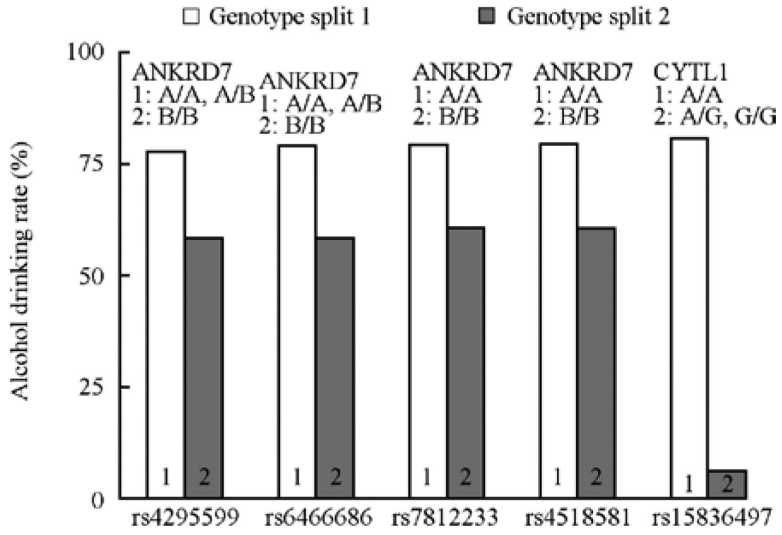

Figure 3.

Alcohol drinking rates stratified by the genotypes of the significant SNPs in the *ANKRD7* and *CYTL1* genes in the total initial Caucasian sample.

Table 1

Characteristics of the subjects of the initial Caucasian GWAS sample

Sex	Age range (years)	Drinkers (*n* (%))	Alcohol consumption levels (%)				
			0	1	2	3	4
Female	19–29 (*n*=80)	69 (86.25)	13.75	68.75	13.75	2.50	1.25
	30–39 (*n*=61)	47 (77.05)	22.95	59.02	14.75	1.64	1.64
	40–49 (*n*=65)	55 (84.62)	15.38	70.77	10.77	0	3.08
	50–59 (*n*=41)	26 (63.41)	36.59	46.34	7.32	7.32	2.44
	60–69 (*n*=121)	80 (66.12)	33.88	45.45	9.92	7.44	3.31
	>=70 (*n*=71)	48 (67.61)	32.39	46.48	7.04	11.27	2.82
	Total (*n*=439)	325 (74.03)	25.97	55.58	10.71	5.24	2.51
Male	19–29 (*n*=86)	68 (79.07)	20.93	43.02	15.12	12.79	8.14
	30–39 (*n*=64)	54 (84.38)	15.63	37.50	25.00	10.94	10.94
	40–49 (*n*=67)	60 (89.55)	10.45	49.25	16.42	13.43	10.45
	50–59 (*n*=37)	32 (86.49)	13.51	45.95	18.92	18.92	2.70
	60–69 (*n*=130)	105 (80.77)	19.23	42.31	13.85	16.15	8.46
	>=70 (*n*=81)	68 (83.95)	16.05	41.98	16.05	14.81	11.11
	Total (*n*=465)	387 (83.23)	16.77	43.01	16.77	14.41	9.03

Table 2

Associations between *ANKRD7* SNPs and AD phenotypes

SNP	Position	Allele*	MAF1†	MAF2‡	Alcohol drinking		Alcohol drinking adjusted by sex and age		Alcohol consumption
					P values	FDR *q* values	*P* values	*OR* (95% *CI*)§	*P* values
rs10249276	118365920	T/C	0.074	0.093	7.99×10^{-7}	0.03	3.73×10^{-6}	0.376 (0.252-0.563)	2.77×10^{-5}
rs10281955	118371927	G/A	0.074	0.092	8.72×10^{-7}	0.02	3.93×10^{-6}	0.377 (0.252-0.564)	2.66×10^{-5}
rs4268066	118388341	C/T	0.075	0.083	3.44×10^{-7}	0.02	2.37×10^{-6}	0.372 (0.249-0.554)	1.04×10^{-5}
rs10953881	118395361	A/G	0.075	0.092	8.21×10^{-7}	0.03	$5.01\text{E}\times10^{-6}$	0.382 (0.256-0.571)	1.65×10^{-5}
rs10953885	118395639	T/C	0.077	0.093	6.63×10^{-7}	0.03	4.03×10^{-6}	0.378 (0.252-0.565)	1.41×10^{-5}
rs4518581	118397891	A/G	0.074	0.093	4.07×10^{-7}	0.02	3.04×10^{-6}	0.373 (0.249-0.558)	1.46×10^{-5}
rs7812233	118405398	G/A	0.074	0.092	8.59×10^{-7}	0.03	5.13×10^{-6}	0.383 (0.256-0.572)	1.63×10^{-5}
rs6466686	118424053	G/A	0.367	0.333	5.86×10^{-8}	0.02	4.35×10^{-7}	0.323 (0.211-0.494)	2.88×10^{-5}
rs4730908	118445246	G/C	0.362	0.333	1.12×10^{-7}	0.02	7.87×10^{-7}	0.325 (0.211-0.501)	3.56×10^{-5}
rs170099	118787448	C/T	0.029	0.050	7.16×10^{-7}	0.03	7.55×10^{-7}	0.210 (0.115-0.385)	3.82×10^{-5}

*The former allele represents the minor allele of each locus.

†Minor allele frequency calculated in our own Caucasian sample.

‡Minor allele frequency reported for Caucasians in the public database of HapMap CEU.

§Odds ratios (*OR*) with 95% confidence intervals (*CI*) are for carriers of the minor allele. *OR* values <1 indicate the protective role of the minor alleles for alcohol drinking behavior.

Table 3

Analysis of the transcript factor binding sites (52 bp) around different alleles at the interested SNP loci of *ANKRD7*

dbSNP ID alleles	With former allele						With latter allele					
	Factor	Strand	Start	End	Score	Eval	Factor	Strand	Start	End	Score	Eval
rs7812233 A/G	RXR-VDR	–	6	19	2.1	11	RXR-VDR	–	6	19	2.1	11
	PPAR-gamma1	–	15	25	5.2	16	PPAR-gamma1	–	15	25	5.2	16
	SP1	–	16	24	3.1	1.3	SP1	–	16	24	3.1	1
	Sp1	–	16	25	3.1	10	Sp1	–	16	25	3.1	10
							REVERB-alpha	–	17	27	4.5	14
	RORalfa-2	–	1	12	0.2	18	RORalfa-2	–	1	12	0.2	18
	POU3F2	–	10	19	4.0	11	POU3F2	–	10	19	4.0	11
							CIZ	–	24	32	3.5	18
							Hunchback	–	25	34	1.7	14
							HMG-IY	–	26	40	1.9	11
	MCM1	+	29	44	5.2	11	MCM1	+	29	44	5.2	11
	MZF_5-13	–	29	37	1.7	12	MZF_5-13	–	29	37	1.7	12
	S8	+	38	44	4.4	13	S8	+	38	44	4.4	13
rs6466686 C/T	Nkx	–	38	44	2.7	6.2	Nkx	–	38	44	2.7	6
	Athb-1	+	39	45	1.9	14	Athb-1	+	39	45	1.9	14
	POU1F1	–	39	48	5.1	9.2	POU1F1	–	39	48	5.1	9
	Lhx3	+	39	48	4.7	15	Lhx3	+	39	48	4.7	15
	Pbx	+	40	49	2.4	10	Pbx	+	40	49	2.4	10
	POU3F2	–	40	52	3.9	19	POU3F2	–	40	52	3.9	19
	Pbx-1a	+	40	48	4.1	17	Pbx-1a	+	40	48	4.1	17
	Pax-2	+	44	50	0.5	20	Pax-2	+	44	50	0.5	20
rs4320489 A/G	POU3F2	–	7	16	3.2	19	POU3F2	–	7	16	3.2	19
	HMG-IY	+	16	30	1.3	16						
	octamer-binding factor	+	21	28	1.5	17						
	NF-A	–	21	28	2.0	16						
	POU2F2	+	21	28	4.5	13						
	CIZ6-1	+	23	37	4.9	18						

dbSNP ID alleles	With former allele						With latter allele					
	Factor	Strand	Start	End	Score	Eval	Factor	Strand	Start	End	Score	Eval
	E2F	–	33	40	1.1	20	E2F	–	33	40	1.1	20
rs170099 A/G	TGA1a	–	4	25	3.9	11	TGA1a	–	4	25	3.9	11
							COMP1	–	18	40	3.8	11
							Gfi	–	19	27	2.0	18
							Pbx	–	20	29	2.1	12
							SRY	–	24	30	1.5	17
	Pbx	+	27	36	5.0	2.8	Pbx	+	27	36	2.1	12
	alpha-CP1	+	33	43	3.5	17	alpha-CP1	+	33	43	3.5	17

Table 4

Replication results of five interested SNPs in *ANKRD7* and *CYTL1* for alcohol consumption trait

Gene	SNP	Initial Caucasian Population (*n*=904)			Caucasian Nuclear Families (*n*=1972)		Unrelated Caucasians (*n*=761)		Chinese Population (*n*=2955)		Combined *P* values[§]
		Allele[*]	MAF	*P* values	MAF	*P* value[†]	MAF	*P* values[†]	MAF	*P* values[‡]	
ANKRD7	rs6466686	G/A	0.36	2.88×10^{-3}	0.37	0.638	–	–	0.30	0.009	2.96×10^{-3}
	rs4295599	T/G	0.24	2.46×10^{-3}	0.25	0.040	–	–	0.30	0.017	5.79×10^{-5}
	rs7812233	G/A	0.07	1.63×10^{-5}	0.07	0.736	0.11	0.808	0.08	0.230	4.49×10^{-2}
	rs4518581	G/A	0.07	1.45×10^{-5}	0.07	0.808	0.11	0.472	0.09	0.375	3.22×10^{-2}
CYTL1	rs16836497	G/A	0.19	2.54×10^{-4}	–	–	0.24	0.030	–	–	7.46×10^{-5}

[*] The former allele represents the minor one of each SNP locus.

[†] *P* values were for alcohol consumption trait adjusted by covariates.

[‡] *P* values were for maximum alcohol consumption.

[§] *P* values were meta-analysis *P* values summarized across the initial GWAS and the follow-up three replication studies