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Sex chromosome-wide association analysis suggested male-specific risk genes for alcohol dependence

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Abstract

Alcohol dependence is more common among men than among women. Potential explanations for this male excess include a role of genes on sex chromosomes (X and Y). In the present study, we scanned the entire Y chromosome and its homologues on X chromosome in males, in order to identify male-specific risk genes for alcohol dependence. Two thousand nine hundred twenty-seven subjects in two independent cohorts were analyzed. The European-American male cohort [883 cases with alcohol dependence and 445 controls] served as the discovery cohort and the European-American female cohort [526 cases and 1,073 controls] served as a contrast group. All subjects were genotyped on the Illumina Human 1M beadchip. Two thousand two hundred twenty-four SNPs on Y chromosome or in the homologues on X chromosome were analyzed. The allele frequencies were compared between cases and controls within each cohort using logistic regression analysis. We found that, after experiment-wide correction, 2 SNPs on the X chromosome were significantly associated with alcohol dependence in European-American males ($p=1.0\times10^{-4}$ for rs5916144 and $p=5.5\times10^{-5}$ for rs5961794 at 3'UTR of *NLGN4X*), but not in females. A total of twenty-six SNPs at 3'UTR of or within *NLGN4X* were nominally associated with alcohol dependence in males ($5.5\times10^{-5}\leq p\leq p0.05$), all of which were not statistically significant in females. We conclude that *NLGN4X* was a significant male-specific risk gene for alcohol dependence in European-Americans. *NLGN4X* might harbor a causal variant(s) for alcohol dependence. A defect of synaptogenesis in neuronal circuitry caused by *NLGN4X* mutations is believed to play a role in alcohol dependence.

Keywords

Alcohol dependence; NLGN4X; Y chromosome; Homologue; Male-specificity; Synaptogenesis

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Introduction

Studies in many cultures have found that the prevalence of alcohol dependence and heavy drinking generally is higher among men than among women (Heath *et al.*, 1998). Potential explanations for this male excess include a role of genes on sex chromosomes (X and Y), or genes on autosomes that are related to the function of sex-related hormones or intrauterine or postnatal hormonal influences. However, the role of sex chromosomes in alcohol dependence has not been investigated comprehensively so far. In the present study, we scanned the entire Y chromosome and its homologues on X chromosome in males, in order to identify male-specific risk genes for alcohol dependence.

Y chromosome comprises two pseudoautosomal regions (PARs) at both ends (5%) and a male-specific region (MSY) in between (95%). Sequences in PARs are the only portions on Y chromosome that are identical (~100%) to the corresponding PARs on X chromosome, reflecting the exchange of DNA between X and Y (crossover) that occurs during sperm production. The PAR1 at Yp11.32/Xp22.33 (2.7Mb) is the result of an obligatory recombination in male meiosis; gene loci in the PAR1 are present in two copies in both males and females and are not subject to dosage compensation by X chromosome inactivation (Helena Mangs and Morris, 2007). These genes in the PAR1 include ASMT, ASMTL, CD99, CRLF2, CSF2RA, SFRS17A, DHRS, GTPBP6, IL3RA, P2RY8, PLCXD1, PPP2R3B, SHOX, SLC25A6, XG and *ZBED1*. The PAR2 at Yq12/Xq28 (330Kb) was created by duplication of material from X to Y since the divergence of human and chimpanzee lineages about 6 million years ago. The genes in the PAR2 include *SPRY3*, *VAMP7*, *SYBL1* and *IL9R*, some of which are subject to X chromosome inactivation (Helena Mangs and Morris, 2007). MSY consists of heterochromatin and euchromatin. Heterochromatin hugs the centromere and the Yq12 region (without PAR2) at the telomere, which are tightly coiled and enriched with repetitive DNA sequences. Heterochromatin maintains the chromosome's structural integrity. It is interspersed with euchromatin along the rest of MSY. All active genes are located in the euchromatin. Euchromatin is classified to X-transposed region (XTR), X-degenerate region (XDR), ampliconic region and others. (1) The X-transposed region (3.38Mb) is located at Yp11.2, next to the PAR1. Its sequences are 99% identical to DNA sequences in Xq21.3 (3.91Mb), a band in the midst of Xq chromosome. This XTR was transposed from X to Y about 6 million years ago, after the divergence of the human and chimpanzee lineages. It escapes X chromosome inactivation and does not require dosage compensation. This X-transposed region contains two known pairs of functional genes, i.e., *PCDH11X* and *PCDH11Y*, a pair of protocadherin genes significantly expressed in adult human brain, and *TGIF2LX* and *TGIF2LY*, a pair of transforming growth factor-beta-induced factor 2 like genes exclusively expressed in testes. This region is hominid-specific and absent from the Y chromosome of non-human primates (Blanco *et al.*, 2000; Williams *et al.*, 2006; Wilson *et al.*, 2006). It might thus play a role in hominid-specific characteristics including the cerebral asymmetry (brain lateralisation), a basis of human-specific cognitive functions such as language (adaptive traits). (2) The X-degenerate segments on Y are not concentrated in one region. They are separated into 12 blocks by some single-copy genes or pseudogenes. But the homologues of these 12 blocks on X chromosome are concentrated in a 6Mb continuous region around Xp22.32, next to the PAR1 (Xp22.33). XDRs display 60%-96% of sequence identity between X and Y. They are surviving relics of ancient autosomes from which the X and Y chromosome co-evolved. XDRs hug SOX3/SRY, RPS4X/Y1, ZFX/Y, AMELX/Y, TBL1X/Y, PRKX/Y, USP9X/Y, DBX/Y, UTX/Y, TMSB4X/Y, NLGN4X/Y, CXorf15/CYorf15A, CXorf15/CYorf15B, SMCX/Y, EIF1AX/Y and *RPS4X/Y2*. Most of these genes are ubiquitous expressed, but only *NLGN4X/Y* are significantly expressed in adult human brain (Lin *et al.*, 2012). (3) The ampliconic regions are non-recombinant and Y-specific; that is, they are single-copy and do not have homologous regions on X. The genes in these regions are exclusively expressed in

testes. Deletions in these regions are a recognized cause of male infertility. That is, the PAR1, XTR and XDR are more likely to be relevant to brain disorders.

In summary, a total of 54 functional homologous genes have been identified so far, including 24 in PAR1, 5 in PAR2, 3 in XTR, 15 in XDR and 7 in other regions. It is estimated that approximately 15 protein-coding genes on the Y chromosome are Y-specific and have no detectable X chromosome homologue. In the present study, all homologous genes on both X and Y chromosomes, in addition to other Y-specific genes on Y, were tested. These homologues are thought to balance their dosage between males and females. Therefore, as contrast, these homologues were also tested in females, in order to see if the risk genes among those homologues are male-specific or shared by females. Because the phasing information of these homologues was not available in the microarray platforms, we focused on individual variants but not multi-locus haplotypes in the present study.

Materials and Methods

Subjects

Two thousand nine hundred twenty-seven subjects in two independent cohorts were analyzed. The European-American male cohort [883 cases with alcohol dependence (DSM-IV) and 445 controls] served as the discovery cohort, and the European-American female cohort [526 cases and 1,073 controls] served as a contrast group. Additionally, two smaller African-American cohorts including a male cohort [428 cases and 169 controls] and a female cohort [253 cases and 339 controls] were also analyzed as contrast groups on an exploratory basis. Affected subjects met lifetime DSM-IV criteria (American Psychiatric Association, 1994) for alcohol dependence. Controls were defined as individuals who had been exposed to alcohol (and possibly to other drugs), but had never become addicted to alcohol or other illicit substances (lifetime diagnoses). Controls were also screened to exclude individuals with major axis I disorders, including schizophrenia, mood disorders, and anxiety disorders. More detailed demographic data were published previously (Bierut *et al.*, 2010; Edenberg *et al.*, 2010; Heath *et al.*, 2011; Zuo *et al.*, 2011a; Zuo *et al.*, 2013a; Zuo *et al.*, 2011b; Zuo *et al.*, 2012; Zuo *et al.*, 2013b). All subjects were de-identified in this study. All subjects were genotyped on the Illumina Human 1M beadchip.

Data cleaning

Subjects with poor genotypic data and questionable diagnostic information, subjects with allele discordance, duplicated IDs, potential sample misidentification, sample relatedness, sample misspecification, gender anomalies, chromosome anomalies (such as aneuploidy and mosaic cell populations), missing race, non-European and non-African ethnicity, population group outliers, subjects with a mismatch between self-identified and genetically-inferred ethnicity, and subjects with a missing genotype call rate ≥2% across all SNPs examined were excluded step-by-step (Zuo *et al.*, 2011a). Furthermore, only the SNPs on Y chromosome and the SNPs in the homologous genes on X chromosome were extracted from the 1M beadchip for association analysis. Because some homologous genes, especially in XDR, were not completely identical in sequence between X and Y chromosomes, some of the SNPs in these genes appeared only on X chromosome. Among these extracted SNPs, those with allele discordance or chromosomal anomalies were excluded. We then filtered out the SNPs with an overall missing genotype call rate ≥2% and the SNPs with minor allele frequencies (MAFs) <0.01 in either European-Americans or Africann-Americans. The SNPs on X chromosome in females and the SNPs in the homologous regions in males that deviated from Hardy-Weinberg Equilibrium ($p<10^{-4}$) within European-American or Africann-American controls were also excluded. This selection process yielded a total of 2,224 SNPs for analysis, including 1,481 in 17 homologous genes on X chromosome only,

480 SNPs in 34 genes on Y chromosome only, and 263 SNPs in the recombinant regions (i.e., XY regions) of both X and Y chromosomes [including 16 genes in the PAR1 (ASMT, ASMTL, CD99, CRLF2, CSF2RA, CXYorf2, DHRSXY, DXYS155E, GTPBP6, IL3RA, P2RY8, PLCXD1, PPP2R3B, SHOX, SLC25A6, XG and *ZBED1*), 3 genes in the PAR2 (*IL9R*, *SPRY3* and *SYBL1*), 2 genes in the XTR (*PCDH11X/Y* and *TGIF2LX/Y*), 12 genes in XDR (AMELX/Y, DDX3X/Y, EIF1AX/Y, NLGN4X/Y, PRKX/Y, RPS4X/Y, SMCX/Y, TBL1X/Y, TMSB4X/Y, USP9X/Y, UTX/Y and *ZFX/Y*) and 1 gene in other region (*NXF2*)].

Association analysis

The allele frequencies were compared between cases and controls within each cohort using logistic regression analysis implemented in the program PLINK (Purcell *et al.*, 2007). Diagnosis served as the dependent variable, alleles served as the independent variables, age and the first 10 principal components served as the covariates. The principal component scores of our samples were derived from all autosomal SNPs across the genome using principal component analysis (PCA) implemented in the software package EIGENSTRAT (Price *et al.*, 2006). Each individual received scores on each principal component. The first 10 principal component scores accounted for >95% of variance. These principal components reflected the population structure of our samples. These PCs serving as covariates in the regression model controlled for the population stratification and admixture effects on association analysis.

The experiment-wide significance levels (α) were corrected for the numbers of effective markers that were calculated by the program SNPSpD (Nyholt, 2004), which is an adjusted Bonferroni correction taking the linkage disequilibrium (LD) structure into account. Approximately 379 and 494 effective SNPs that had $r^2 \leq 0.1$ between any two of them captured most of the information of all variants examined in cohorts of European and African descent, respectively. Thus, the corrected significance levels (α) for association tests were set at 1.3×10^{-4} in cohorts of European descent and 1.0×10^{-4} in cohorts of African descent, respectively.

Power analysis

We computed the power of test using the R package “pwr” that uses the effect sizes and the notations from Cohen (1988) (Cohen, 1988). Given the effect size (w), the total number of observations (n), the degree of freedom (df) and the significance level (α), the statistical power of test can be determined from these parameters. The effect size is w=∑[(O-E)^2/E], where O is the observed allele frequency of the targeted SNP and E is its expected allele frequency (see **Table 1**). The α was corrected for multiple testing as described above.

Results

We found 79 (62 on X, 4 on Y, 13 on XY) and 132 (50 on X, 73 on Y, 9 on XY) SNPs were nominally associated with alcohol dependence in European-American males and Africann-American males, respectively ($p<0.05$), and 51 (37 on X and 14 on XY) and 69 (62 on X and 7 on XY) SNPs were nominally associated with alcohol dependence in European-American females and Africann-American females, respectively ($p<0.05$). After experiment-wide correction, 2 SNPs on X were significantly associated with alcohol dependence in European-American males ($p=1.0\times10^{-4}$ for rs5916144 and $p=5.5\times10^{-5}$ for rs5961794 at 3'UTR of *NLGN4X*), but not in other cohorts.

Given the allele frequencies discovered in European-American male cohort listed in Table 1, w = 0.167, df = 1, and $\alpha=10^{-4}$, the European-American female cohort (n=1599) had 99.8% of power to detect the two most significant risk markers. However, the African-American

male and female cohorts only had 68.1% and 51.4% of power to significantly detect them, respectively; and thus, the findings in African-American cohorts were considered exploratory.

We found that 26 out of 444 SNPs at 3'UTR or within *NLGN4X* were nominally associated with alcohol dependence in European-American males ($5.5\times10^{-5}\leq p\leq p0.05$), including those two significant ones. All of these associations were not statistically significant in European-American females, and most of them were not significant in African-Americans males and females either (Table 1).

Discussion

We found that *NLGN4X* on X chromosome was a significant male-specific risk gene for alcohol dependence in European-Americans. *NLGN4X* might harbor a causal variant(s) for alcohol dependence.

NLGN4X, spanning 338 kb, maps to Xp22.32 on XDR, proximal to the PAR1 of X chromosome. It contains six exons and codes for neuroligin 4 (with 816 amino acids) (Ichtchenko *et al.*, 1996). Its homologue on Y chromosome, i.e., *NLGN4Y* (at Yq11.221), is an X-degenerate gene. Forty million years ago, during the process of divergence of Old World from New World Monkeys, *NLGN4X* and *NLGN4Y* were introduced into X and Y chromosomes, respectively, originating from the common ancient autosomes (de Oliveira *et al.*, 2012).

Females have two Xs and males have one X and one Y. To make the gene dosage equivalent between both sexes, females inactivate almost an entire X chromosome. However, about 15% of the genes on the inactive X chromosome, mostly situated on the short arm (Xp) including PAR1 and XDR, permanently escape inactivation (Carrel and Willard, 2005). Consistent with the dosage compensation rule, these 15% of genes have homologues on Y chromosome, so that their dosages are still equivalent between males and females. *NLGN4X* is among these 15% of genes; therefore, *NLGN4X* and *NLGN4Y* are supposed to be paired, diploid-acting genes on chromosomes X and Y, respectively. However, not like the genes in the PARs and XTRs that have sequence identity ≥ 99%, *NLGN4X* and *NLGN4Y* are XDR genes and are approximately 97.5% similar at the amino acid level and less similar at the DNA sequence level (≤96%) (Jamain *et al.*, 2003), so that some variants at *NLGN4X* are located on X chromosome only (although most variants at *NLGN4Y* are located on both X and Y chromosomes according to dbSNPs). Interestingly, all SNPs at *NLGN4X* significantly or nominally associated with alcohol dependence in males identified in the present study are located on single copy of X (Table 1). Those *NLGN4* variants located on both X and Y chromosomes were not associated with alcohol dependence in males. In females, all of the *NLGN4X* markers located on two copies of X chromosomes were not associated with alcohol dependence either (after correction). That is, only haploid *NLGN4X* variants on X in males showed associations with alcohol dependence. Those diploid *NLGN4* SNPs paired between two X chromosomes (in females) or between X and Y chromosomes (in males) were not associated with alcohol dependence.

Two copies of *NLGN4X* in females escape the inactivation and thus both express protein product. When one of the pair has a defective effect, another copy might compensate the defective effect of the risk copy, and thus might protect against diseases. Similarly, when the variants are paired between *NLGN4X* and *NLGN4Y* in males, the defective effects of risk alleles could be compensated too. Only when the defective effects come from the single-copy of X (i.e., in males), could these effects be invariably expressed, increasing risk for diseases, which was exactly what we observed in this study. This is why many male-

dominant diseases are caused by genes on X chromosome, and males are more likely to have X chromosome-related diseases (Zinn *et al.*, 2007).

The male-specific associations between *NLGN4X* and alcohol dependence are paralleled by male dominant inheritance for other disorders where *NLGN4X* is implicated, particularly autism (Jamain *et al.*, 2003). *NLGN4X* is also named as autism susceptibility X-linked type 2 gene (*AUTSX2*). Given the significant phenotypic overlap with autism and the putative role for synaptic plasticity in the etiology of alcohol dependence, it is understandable that *NLGN4X* is also a risk gene for alcohol dependence. Interestingly, other genes have ever linked the heritable risks for alcoholism and autism. For example, the autism susceptibility candidate gene 2 (*AUTS2*) was reported to be a risk gene for alcohol consumption by a genome-wide association study (Schumann *et al.*, 2011). In addition to autism and alcohol dependence, *NLGN4X* has also been associated with several other cognitive disorders including Asperger syndrome, Tourette syndrome, mental retardation, selective nonverbal deficits (e.g., impaired visual-spatial abilities), learning disability and/or schizophrenia (Jamain *et al.*, 2003; Laumonnier *et al.*, 2004; Lawson-Yuen *et al.*, 2008; Walsh *et al.*, 2008; Yan *et al.*, 2005; Zinn *et al.*, 2007).

The brain processes information by transmitting signals at synapses, which connect neurons into vast networks of communicating cells. In these networks, synapses not only transmit signals but also transform and refine them. All processing of information in the brain involves synapses, and almost all abnormalities in brain function have a direct or indirect effect on synaptic function. Neurexins and neuroligins are enriched specifically in the membrane of glutamatergic synapses, especially in the dendritic spines in the hippocampal neurons (Chih *et al.*, 2005). They are arguably the best-characterized synaptic cell-adhesion molecules, and they are the only ones for which a specifically synaptic function has been established (Craig and Kang, 2007; Dean and Dresbach, 2006). Neuroligin–neurexin complexes connect presynaptic and postsynaptic neurons at synapses, mediate signaling across the synapse, and shape the properties of neural networks by specifying synaptic functions, performing a central role in the brain's ability to process information during nervous system development. Neuroligin deficiency in the brain caused by *NLGN4X* mutations may lead to abnormal development of synaptic structures and may have dramatic effects on communication processes and cognitive development; thus, neuroligin is a key target in the pathogenesis of cognitive diseases (Sudhof, 2008).

Imbalance between neuronal excitation and inhibition could lead to epilepsy; the prevalence of epilepsy among alcoholics is at least triple that in the general population (Chan, 1985). It is hypothesized that at least some forms of alcohol dependence might be caused by an imbalance of neuronal excitation/inhibition. Neuroligin 4 is localised to excitatory glutamatergic axons and has a role in adjusting the balance between excitatory and inhibitory synapses (Chih *et al.*, 2005; Graf *et al.*, 2004; Prange *et al.*, 2004). Neuroligin defects caused by *NLGN4X* mutations may abolish formation, stabilization or recognition of specific synapses essential for the communication processes, and subsequently lead to selective loss of excitatory function and abnormal excitatory/inhibitory balance in neurons (Jamain *et al.*, 2003). Such a defect of synaptogenesis in neuronal circuitry is believed to play a role in alcohol dependence.

NLGN4X conferring risk for alcohol dependence might be population-specific (i.e., European-Americans only). However, this specificity is warranted to be confirmed in the future by expanding the sample sizes of African-American cohorts to reach sufficient study power.

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Table 1

Associations between *NLGN4X* gene and alcohol dependence

SNP	Position	Location	Risk allele	Risk allele frequency		Association			Contrast groups		
				Cases	Controls	OR	95%CI	p (EAM)	p (EAF)	p (AAM)	p (AAF)
rs5915821	4746402	3′UTR	T	0.532	0.442	1.45	1.14-1.80	0.001	0.574	0.166	0.012
rs5915826	4752475	3′UTR	A	0.532	0.442	1.45	1.14-1.80	0.001	0.575	0.177	0.006
rs5915829	4758997	3′UTR	G	0.646	0.562	1.43	1.13-1.80	0.002	0.901	0.854	0.199
rs4826671	4771654	3′UTR	C	0.306	0.232	1.51	1.13-1.90	0.002	0.205	0.450	0.550
rs17305063	5396913	3′UTR	G	0.822	0.769	1.40	1.05-1.83	0.017	0.684	0.699	0.313
rs5916106	5440260	3′UTR	A	0.827	0.771	1.43	1.07-1.87	0.012	0.588	0.803	0.446
rs5916133	5464521	3′UTR	T	0.826	0.776	1.40	1.04-1.82	0.021	0.548	0.580	0.288
rs1564673	5486848	3′UTR	G	0.686	0.586	1.58	1.22-1.95	1.5×10^{-4}	0.762	0.527	0.366
rs5916144	5489611	3′UTR	A	0.731	0.633	1.62	1.24-2.01	1.0×10^{-4}	0.870	0.614	0.182
rs5915572	5500890	3′UTR	G	0.740	0.662	1.48	1.13-1.85	0.002	0.770	0.568	0.195
rs5916157	5503597	3′UTR	G	0.689	0.625	1.35	1.05-1.69	0.013	0.814	0.424	0.794
rs5961740	5509538	3′UTR	G	0.689	0.626	1.35	1.04-1.68	0.015	0.811	0.418	0.922
rs4826792	5518048	3′UTR	A	0.729	0.668	1.35	1.05-1.71	0.016	0.508	0.243	0.913
rs5916176	5525522	3′UTR	A	0.706	0.648	1.32	1.03-1.66	0.024	0.595	0.474	0.546
rs5916183	5568175	3′UTR	T	0.481	0.423	1.31	1.01-1.59	0.022	0.808	0.939	0.856
rs9698745	5573674	3′UTR	C	0.725	0.640	1.50	1.16-1.89	0.001	0.407	0.656	0.275
rs7051992	5576009	3′UTR	T	0.720	0.636	1.49	1.16-1.88	0.001	0.412	0.548	0.262
rs5961794	5584328	3′UTR	A	0.721	0.614	1.64	1.28-2.06	5.5×10^{-5}	0.245	0.511	0.792
rs882145	5597394	3′UTR	A	0.631	0.563	1.31	1.05-1.67	0.021	0.412	0.628	0.279
rs2369265	5600519	3′UTR	G	0.632	0.564	1.31	1.05-1.67	0.022	0.408	0.738	0.328
rs11094800	5621715	3′UTR	G	0.490	0.429	1.30	1.02-1.61	0.027	0.659	0.701	0.171
rs5961840	5712122	3′UTR	C	0.798	0.741	1.40	1.06-1.81	0.014	0.437	0.031	0.255
rs5961851	5722793	3′UTR	A	0.802	0.741	1.43	1.08-1.85	0.009	0.617	0.059	0.904
rs5961861	5738176	3′UTR	T	0.674	0.609	1.36	1.05-1.68	0.010	0.961	0.288	0.162
rs16997675	5952368	intron4	G	0.201	0.145	1.44	1.09-2.02	0.022	0.919	0.689	0.242
rs7052989	5954178	intron4	A	0.202	0.150	1.39	1.05-1.94	0.037	0.836	0.237	0.523

OR, odds ratio; p, p-value; EAM, European-American males; EAF, European-American females; AAM, African-American males; AAF, African-American females. Other protein-coding gene closest to these markers, i.e., *PRKX*, is 2,176Kb far away from the 3’ of *NLGN4X*.