Genetic underpinnings of the sex bias in autism spectrum disorder

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SUMMARY
Autism spectrum disorder (ASD) is a neurodevelopmental condition featuring difficulties in social interaction and communication. ASD is also well-known for its sex bias, in which males are diagnosed approximately four times more frequently than females. Although recent sequencing studies have identified a genetic etiology of ASD, the sex bias in ASD still remains largely unexplained because the frequencies of autosomal ASD-associated genetic variations are equivalent between males and females. We hypothesized that if the intrinsic expression levels of ASD-associated genes are different between males and females, they may contribute to the sex bias in ASD. Aiming to decode the genetic contribution to the ASD sex bias, we determined whether gene expression levels of ASD-associated genes were different between undiagnosed males and females. Analyses of the ribonucleic acid sequencing (RNAseq) data from the Genotype-Tissue Expression consortium (GTEx) revealed modest differences for 17 ASD-associated genes in the relevant brain regions between males and females. These candidate genes showed stronger collective sex-dependent expression in the gene set enrichment analysis, which was subsequently validated by the RNAseq data from the PsychENCODE consortium. In addition, gene ontology analysis revealed the functional relevance of the 17 candidate genes in chromatin remodeling and synaptic regulation. Taken together, these candidate genes discovered through big data analysis suggest that sex-dependent expression of ASD-associated genes may contribute to the sex bias in ASD. These novel insights into the sex bias may refine underlying disease mechanisms of ASD, potentially informing therapeutic strategies for improving the quality of life of diagnosed individuals.

INTRODUCTION
Autism Spectrum Disorder (ASD) represents a neurodevelopmental condition involving difficulties in social interaction and repetitive behaviors [1]. The overall prevalence of ASD is 2.7% in the US, indicating that ASD is quite common [2; 3]. Clinical characteristics of ASD are highly heterogeneous [4-6]. For example, some individuals with ASD may display impaired intellectual disability, while others display higher intelligence quotients [7]. A better understanding of such variances in the phenotypic presentation among autistic individuals has led to improvements in diagnostic practices [8]. However, effective treatments or prevention for ASD have yet to be developed, and treatments for ASD are limited to mitigating symptoms [9]. Since genetically supported targets significantly increased the success rates of clinical trials, genes underlying ASD may represent excellent therapeutic targets [10]. Importantly, family-based genetic studies showed a high heritability in ASD, attributing 40-80% of ASD susceptibility to genetics [2; 11]. In support, large-scale genetic studies demonstrate that common genetic variations contribute to the etiology of ASD [12; 13].

Although the discovery of ASD-associated genes significantly widened the field’s knowledge of ASD, these genes could not explain many important aspects of ASD. One such phenomenon is sex bias, in which boys are approximately four times more likely to be diagnosed with ASD compared to age-matched girls [14; 15]. Given the striking sex bias and significant genetic contribution in ASD, sex-specific factors have been speculated to produce this phenomenon [12; 13; 16; 17]. For instance, sex-linked genes and/or sex hormones have been proposed to contribute to the sex bias in ASD [14]. However, genetic studies revealed only a handful of significantly associated sex-chromosome single nucleotide polymorphisms (SNPs) in ASD [18; 19]. In contrast, recent large-scale genetic association studies discovered numerous autosomal SNPs that are significantly associated with ASD [12; 16]. Together, these data suggest that ASD-associated SNPs genes on the autosomes may play an important role in the sex bias in ASD. Yet, the mechanism underlying how autosomal SNPs can contribute to the sex bias in ASD is unclear given their allele frequencies are equal between males and females and the resulting altered protein function is expected to be the same between sexes.

To explain the sex bias in ASD, we hypothesized that differences in the expression levels of ASD-associated genes between males and females play an important role in the sex bias in ASD. For example, the sex bias in ASD could be explained if 1) ASD-associated SNPs change the expression levels of corresponding ASD-associated genes in males and females differently and/or 2) ASD-associated SNPs alter the function of corresponding genes whose expression levels are intrinsically different between males and females. Since understanding the genetic underpinnings of sex bias may shed light on the biological mechanisms of ASD and, therefore, inform novel therapeutic strategies for ASD, we set out to investigate a role for sex-dependent expression of ASD-associated genes in the sex bias of ASD.

Recently, a large-scale genetic study revealed 102 genes
that were significantly associated with ASD, providing a genetic landscape of ASD [16]. Here, we determined whether the intrinsic expression levels of autosomal ASD-associated genes were different in the relevant brain regions (e.g., amygdala, cortex, frontal cortex, and anterior cingulate cortex) between presumably undiagnosed males and females (20-70 years old) to gain insights into the genetic underpinning of the sex bias in ASD [20-22]. Through analysis of RNAseq data from ASD-relevant brain regions, we found that 17 ASD-associated genes are differentially expressed between males and females, suggesting they may play a role in the genetically-driven sex bias in ASD. These findings may open the door for additional genetic investigations to understand underlying mechanisms of ASD.

RESULTS
Identification of ASD-Associated Genes whose Expression Levels are Intrinsically Different between Males and Females
To test an alternative hypothesis that can explain the significant sex bias in ASD, we investigated whether the expression levels of 102 genes that were reported as ASD-associated genes in a recent large-scale genetic study had different expression levels in presumably undiagnosed males and females using two large public genome-wide expression data sets such as Genotype-Tissue Expression (GTEx) and PsychENCODE (PEC) RNAseq data sets (20-70 years old) [16; 21; 22]. To determine whether the expression levels of ASD-associated genes are different between non-autistic males and females, we analyzed GTEx RNAseq data [21], which comprised presumably undiagnosed individuals. Linear regression analysis focusing on the 102 ASD-associated genes revealed 18 genes in the amygdala, 15 genes in the cortex (right cerebral frontal pole cortex), 15 genes in the frontal cortex (right cerebral frontal pole cortex), and 14 genes in the anterior cingulate cortex that had significantly different expression between males and females (Table 1). A total of 21 different ASD-associated genes showed significant expression differences between males and females in at least one tissue (Table 1). To increase confidence, we used ASD-associated genes that showed significant p-values in at least two brain regions as candidate sex-specific ASD-associated genes for subsequent analyses. Among 102 ASD-associated genes, 17 genes (ADNP, DYRK1A, SHANK3, TLK2, PTK7, MKX, STXBP1, TCF4, DYSPLS2, NSD1, PHF2, KCNQ3, SETD5, RAII, SKI, ZMYND8, and NACC1) showed at least nominally significant sex differences in at least two brain regions (Figure 1). Clearly, not all ASD-associated genes were differently expressed between presumably undiagnosed males and females. However, more than 15% of the 102 ASD-associated genes had different expression levels between males and females, which was greater than expected based on the statistical threshold that we used (5% were expected to be significant based on \( p < 0.05 \)). Together, these data indicated that some ASD-associated genes are expressed differently in presumably undiagnosed males and females, supporting their potential roles in the sex bias in ASD.

<table>
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Table 1: ASD-associated genes that showed different expression levels between undiagnosed males and females in at least one brain region of GTEx data. We statistically modeled the expression levels of each ASD-associated gene as a function of sex and other covariates to determine whether ASD-associated genes are expressed differently in males and females in ASD-relevant brain regions using the GTEx data set (amygdala, n = 129; cortex, n = 205; frontal cortex, n = 175; anterior cingulate cortex, n = 147). Twenty-one ASD-associated genes showed nominally significant p-values for sex in at least one brain region. The table displays sex p-values and effect size (beta) for each of the 21 genes. A positive beta means increased expression level in females. Yellow highlight indicates a statistical significance (\( p < 0.05 \)).
Enrichment of Candidate ASD-Associated Genes in the Genes with Sex-Dependent Expression

Considering the limitations of individual gene analysis of small sample sizes in the GTEx data, ASD-associated genes showed modest effects and significance levels for sex, as anticipated (Figure 1). To overcome the limitation of single gene analysis and the burden of multiple test correction, we performed gene set enrichment analysis (GSEA) of ASD-associated genes. GSEA analysis showed that the set of 102 ASD-associated genes was not significantly enriched in the sex-dependent genes in any of the relevant brain regions (Figure 2). In contrast, the 17 previously identified ASD-associated genes, which were nominally significant in the individual gene analysis, showed a significant enrichment in the sex-dependent genes in any of the relevant brain regions (Figure 2). Similar to single gene analysis, these data indicated that not all ASD-associated genes are expressed differently between males and females. Rather, significantly different expression levels of 17 ASD-associated genes in males and females implied that they may play a role in the sex bias in ASD.

Validation of 17 Candidate Sex-Dependent ASD-Associated Genes

Discovery data analysis using the GTEx data showed that 17 ASD-associated genes were differently expressed between males and females (i.e., sex-dependent ASD-associated genes), informing potential mechanisms by which autosomal ASD-associated genes contribute to the sex bias in ASD. To further confirm the significant enrichment of the 17 candidate ASD-associated genes in the genes with sex-dependent expression, we validated our findings using an independent data set. For this, we analyzed the PEC RNAseq data for the prefrontal cortex. The PEC Consortium has a collective goal of accelerating discoveries of functional genomic elements in the human brain and elucidating their roles in the molecular pathophysiology of psychiatric disorders [22]. RNAseq data produced by the PEC Consortium offer increased power due to a large sample size (n = 1,435). However, the PEC RNAseq data analyzed only one tissue (prefrontal cortex). Similar to GTEx data analysis, we performed linear regression analysis for all transcripts to generate a complete list of sex-dependent genes in the PEC data. Subsequently, gene set enrichment analysis for the 17 candidate genes was performed to identify enrichment for sex-associated genes. The set of 17 ASD-associated genes was significantly (p = 0.00022) enriched in the sex-dependent genes in the PEC data, supporting sex-dependent expression of the 17 candidate ASD-associated genes (Figure 4).

Pathway Analysis of Sex-Dependent ASD-Associated Genes

Discovery and validation analyses revealed that the expression levels of 17 ASD-associated genes were significantly different between males and females. To gain insights into how altered expression levels and/or functions of sex-dependent ASD-associated genes may lead to sex bias in ASD, we performed gene ontology pathway analysis [23; 24]. The 17 identified genes showed significant fold-enrichment and statistical significance (false discovery rate < 0.05) for biological pathways related to synaptic function and chromatin regulation, suggesting that males and females may have different signaling in those pathways (Figure 5). Taken together, these data may support a role for neuronal function and gene expression regulation in the diagnosis of ASD in a sex-dependent manner, suggesting a role for autosomal ASD-associated genes in the sex bias in ASD.

DISCUSSION

Understanding the mechanisms that are responsible for the sex bias in ASD is important because such knowledge will shed light on the fundamental biology of ASD and thus, may inform therapeutic strategies for autistic individuals. There is evidence that the disproportionality of male ASD diagnoses is due to diagnostic bias and genetic
predispositions. Specifically, it has been shown females are less likely diagnosed with ASD compared to males [14; 15]. The sex bias in ASD may be partially explained by diagnostic bias as females are better able to hide or compensate for their difficulties [25]. In addition, sex chromosomes and sex hormones were implicated in the sex bias in ASD as ASD diagnosis was increased in genetic conditions with abnormal sex chromosomes such as Turner syndrome (X0) and Klinefelter syndrome (XXY) [26-28]. However, X chromosome trisomy (XXX) also increased the prevalence of ASD, suggesting a possibility that the Y chromosome is a risk factor, while the second X chromosome is a protective factor [29; 30]. In addition, fetal testosterone, the levels of androgen, and increased serum androstenedione were hypothesized to be involved in the sex bias in ASD [31-35]. These data indicate that abnormalities in sex chromosomes and levels of sex hormones may contribute to biased prevalence in ASD. However, the majority of ASD cases do not have abnormal sex chromosomes, males and females may express similar levels of genes on the chromosome X due to X-inactivation, and large-scale genetic analyses failed to detect a significant association between ASD and SNPs on genes encoding sex hormones [12; 16]. Therefore, these data support the existence of additional factors that contribute to the sex bias in ASD, such as sex-dependent autosomal variations.

Since common genetic variations account for the majority of heritability of ASD, we hypothesized that the same genetic variations associated with ASD might also contribute to its sex bias [13]. Interestingly, many genes on the autosomes are significantly associated with ASD, supporting a role for ASD-associated autosomal genes in the sex bias in ASD [16]. Still, the sex bias could not be explained by those significantly associated autosomal SNP variations per se because their frequencies are equal between males and females. However, if the ASD-associated genes are intrinsically expressed differently between males and females, genetic variations might contribute to the diagnosis of ASD in a sex-dependent manner. For example, if ASD is caused by significantly reduced

Figure 2: Testing the enrichment of 102 ASD-associated genes in the sex-differentially expressed genes in the GTEx data. We performed gene set enrichment analysis to test whether 102 ASD-associated genes were enriched as a set in the genes showing sex-dependent expression levels in the test brain regions in the GTEx data such as A) amygdala, B) cortex, C) frontal cortex, and D) anterior cingulate cortex. We calculated the gene set score of 102 genes (red triangles) and compared to the null distributions of the gene set score of the randomly selected 102 genes, which were based on 100,000 permutations. Brain regions and p-values are displayed at the top right corners.
function of an autosomal gene 'A', which is expressed lower in males normally, genetic variations that further decrease the expression levels and/or its activity are expected to produce increased risk for ASD in males compared to females. We therefore determined whether expression levels of ASD-associated genes [16] were different between males and females in ASD-relevant brain regions including the amygdala, cortex, frontal cortex, and anterior cingulate cortex [20].

To answer this question, we analyzed publicly available large data sets such as GTEx and the PEC RNAseq data set. Initial discovery analysis using the GTEx data revealed that only 17 of 102 ASD-associated genes showed significant expression level differences between males and females in at least two ASD-relevant brain regions. Importantly, those 17 candidate genes showed significant enrichment in the list of total sex-biased genes in the GTEx data, which was further validated in the independent PEC RNAseq data. The significant enrichment of 17 candidate genes may suggest the importance of collective effects because ASD-associated genes individually generate modest effects, but they might produce significant effects together [12; 13; 36; 37]. Importantly, gene ontology analysis of 17 candidate genes indicated that these genes were involved in synaptic function or chromatin regulation, and thus, these processes may be different between males and females. This is consistent with roles for those pathways in ASD as synaptic, transcriptional and chromatin genes are disrupted in autism [38].

In summary, our genetic investigation focusing on the sex bias in ASD revealed an intriguing possibility that intrinsic sex-dependent differences in the expression levels of candidate ASD-associated genes may contribute to high incidence of ASD in males. Previously, the importance of the expression levels of ASD-associated genes has been demonstrated in mouse models. For example, SHANK3 is one of the ASD-associated genes that showed significant differences in expression levels between males and females. SHANK3

Figure 3: Testing the enrichment of 17 candidate ASD-associated genes in the sex-differentially expressed genes in the GTEx data. We performed gene set enrichment analysis to test whether 17 candidate ASD-associated genes were enriched as a set in the genes showing sex-dependent expression levels in the test brain regions in the GTEx data, including the A) amygdala, B) cortex, C) frontal cortex, and D) anterior cingulate cortex. We calculated the gene set score of 17 candidate genes (red triangles) and compared to the null distributions of gene set score of the randomly selected 17 genes, which were based on 100,000 permutations. Brain regions and p-values are displayed at the top right corners. Significant p-values indicated that a set of 17 ASD-associated genes were enriched in the sex-dependent genes, suggesting that 17 candidate ASD-associated genes are differently expressed between males and females.
knock-out mice displayed autistic-like behaviors, supporting that expression levels of SHANK3 play an important role in ASD [39]. This example supported a role for a single gene in ASD and its sex bias. Alternatively, collective effects due to modestly altered expression levels of many ASD-associated genes might be responsible for sex bias in ASD, which remains to be tested. In addition, our results were based on computational data analysis, representing one of the limitations. Therefore, subsequent experimental validations using patient-derived cells and/or genetically-modified mouse models are required to directly test our proposition. Nevertheless, our data demonstrating the differences in the levels of ASD-associated genes between non-autistic males and females may provide new directions for therapeutics such as targeting gene expression levels. Taken together, our data suggested a role for intrinsic expression level differences in ASD-associated genes in contributing to the sex bias in ASD. Thus, our study may facilitate uncovering fundamental aspects of ASD that can be targeted as part of precision medicine for autistic individuals.

Figure 4: Validation of 17 sex-differentially expressed ASD-associated genes. A) We performed linear regression analysis to calculate p-values and effect size for each candidate gene (red circles) using PEC data (n = 1435). Statistical significance (y-axis; -log₁₀(p-value)) was compared to the effect size (x-axis). B) We performed gene set enrichment analysis of PEC data focusing on 17 candidate genes by comparing the true gene set score to a distribution of scores of random gene set. The gene set score (red triangle) was compared to the null distribution of gene set score of random gene sets. p-value is displayed at the top right corner. The 17 candidate genes were significantly enriched in the PEC data, validating the set-wide significance of the candidate genes.

Figure 5: Significantly enriched biological pathways in sex-differentially expressed ASD-associated genes. We performed gene ontology analysis for the candidate genes to identify significantly enriched biological processes in the sex-dependent ASD-associated genes. The colored bars reflect the levels of significance.
MATERIALS AND METHODS

Discovery Analysis to Test Sex-Dependent Expression of ASD-Associated Genes using the GTEx Data

GTEx project is an ongoing effort to build a comprehensive public resource to study tissue-specific gene expression and regulation [21]. Various tissues were analyzed by the RNA-Seq platform by the GTEx consortium, offering expression levels of 24,718 genes in 44 different tissues [21]. The range of participants' ages was 20-70. In this project, we analyzed RNAseq data for brain regions that are relevant in ASD such as the amygdala (n = 129; 92 males and 37 females), cortex (n = 205; 141 males and 64 females), frontal cortex (n = 175; 127 males and 48 females), and anterior cingulate cortex (n=147; 105 males and 42 females). We downloaded the expression data of 102 ASD-associated genes from the GTEx portal version 8 [21]. In single gene analysis, we determined whether the expression level of a given gene was significantly different between males and females. Briefly, we performed linear regression analysis using the R program for each of 102 ASD-associated genes to explain its expression level as a function of sex as the main predictor variable and a set of covariates (values from principal component analysis representing characteristics of samples), which were downloaded from the GTEx portal website. Since the size of samples in each tissue in the GTEx data set is relatively small (< 300), we applied a nominal significance threshold (p < 0.05) to identify genes differently expressed between males and females to minimize false negatives.

Gene Set Enrichment Analysis of ASD-Associated Genes using GTEx Data

We performed a single test to determine whether ASD-associated genes as a set were significantly enriched in the sex-differentially expressed genes. For this, we carried out gene set enrichment analysis (GSEA) using the R program [40]. Specifically, we performed linear regression analysis to determine the significance level of sex in each gene. Then, we sorted the GTEx analysis results based on the significance value (−log10(p-value)) of the sex, and subsequently tested whether sets of ASD-associated genes (102 and 17 genes) were enriched at the top of sex-differentially expressed genes in the GTEx analysis. We calculated the significance of enrichment by comparing the sum of significance values (−log10(p-value)) of ASD-associated genes (i.e., true set gene set score) to the distribution of randomly selected gene set values of the same gene numbers (i.e., null distribution of the gene set score). The distribution of random selections was based on 100,000 random permutations. When the number of random gene sets whose scores were greater than the true gene set score did not exceed 5,000, it was empirically considered statistically significant (p < 0.05).

Validation Analysis using the PEC Data

To validate the findings of GSEA analysis of sex-dependent ASD-associated genes in the GTEx data with increased power, we also analyzed the PEC RNAseq data. The PEC Consortium has a collective goal of accelerating discoveries of functional genomic elements in the human brain and elucidating their roles in the molecular pathophysiology of psychiatric disorders [22]. RNAseq data produced by the PEC Consortium offer increased power due to a large sample size (n = 1,435). However, the PEC RNAseq data analyzed only one tissue (prefrontal cortex). The PEC RNAseq data for the replication analysis were downloaded from the PEC Knowledge Portal [22]. To validate of the enrichment of the 17 ASD-associated genes in the sex-dependent genes using the PEC data, we performed GSEA as described in the GTEx analysis section.

Gene Ontology Pathway Analysis

We input the 17 candidate sex-specific ASD-associated genes into the gene ontology analysis program, which is powered by PANTHER [23; 41]. We selected the 'biological processes' and 'Homo sapiens' options to identify pathways that were enriched in the candidate ASD-associated genes. We considered pathways that generated a false discovery rate less than 0.05 as significant.

Multiple Test Correction

Since the GTEx dataset was smaller, the identification of candidate ASD-associated genes was based on nominal p-values. We did not correct the p-value from the gene set enrichment analysis because a single test was performed. We corrected the significance of Gene Ontology analysis by using false discovery rate. We used R version 4.1.0 for all statistical analyses.

REFERENCES

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