Molecular Psychiatry (2011) 16, 949–959

www.nature.com/mp

ORIGINAL ARTICLE

A genome-wide association study on common SNPs and rare CNVs in anorexia nervosa

K Wang[1], H Zhang[1], CS Bloss[2], V Duvvuri[3], W Kaye[3], NJ Schork[2,4], W Berrettini[5], H Hakonarson[1,6], and the Price Foundation Collaborative Group[7]

[1]*Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, PA, USA;* [2]*The Scripps Translational Science Institute, Scripps Health, La Jolla, CA, USA;* [3]*Department of Psychiatry and Eating Disorders Treatment and Research Program, University of California San Diego, La Jolla, CA, USA;* [4]*Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA, USA;* [5]*Department of Psychiatry, University of Pennsylvania, Philadelphia, PA, USA and* [6]*Department of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA*

Anorexia nervosa (AN) is a mental illness with high mortality that most commonly afflicts adolescent female individuals. Clinical symptoms include chronic food refusal, weight loss and body image distortions. We carried out a genome-wide association study on 1033 AN cases and 3733 pediatric control subjects, all of whom were of European ancestry and were genotyped on the Illumina HumanHap610 platform (Illumina, San Diego, CA, USA). We confirmed that common single-nucleotide polymorphisms (SNPs) within *OPRD1* (rs533123, $P=0.0015$) confer risk for AN, and obtained suggestive evidence that common SNPs near *HTR1D* (rs7532266, $P=0.04$) confer risk for restricting-type AN specifically. However, no SNPs reached genome-wide significance in our data, whereas top association signals were detected near *ZNF804B*, *CSRP2BP*, *NTNG1*, *AKAP6* and *CDH9*. In parallel, we performed genome-wide analysis on copy number variations (CNVs) using the signal intensity data from the SNP arrays. We did not find evidence that AN cases have more CNVs than control subjects, nor do they have over-representation of rare or large CNVs. However, we identified several regions with rare CNVs that were only observed in AN cases, including a recurrent 13q12 deletion (1.5 Mb) disrupting *SCAS* in two cases, and CNVs disrupting the *CNTN6*/*CNTN4* region in several AN cases. In conclusion, our study suggests that both common SNPs and rare CNVs may confer genetic risk to AN. These results point to intriguing genes that await further validation in independent cohorts for confirmatory roles in AN.
Molecular Psychiatry (2011) **16,** 949–959; doi:10.1038/mp.2010.107; published online 16 November 2010

Keywords: anorexia; eating disorder; copy number variation; genome-wide association study; OPRD1; HTR1D

Introduction

Anorexia nervosa (AN) is a syndrome characterized by chronic food refusal, weight loss, irrational fear of weight gain even when emaciated, and distortions about body weight and shape.[1] Women are affected more often than men in an ~10:1 ratio, with ~0.9% of US women affected and a near uniform adolescent age at onset.[2] There are several subtypes of AN.[1] Those with the restricting subtype have severely diminished food intake, and those with the binge-eating/purging subtype additionally engage in binge-eating or purging behavior. Compared with other behavioral disorders, AN has a stereotypic presentation with respect to gender-specific risk, age of onset, symptoms, signs and disease course.[2] AN is associated with significant morbidity and has the highest mortality rate among all psychiatric disorders,[3] with recent estimates of a standardized mortality ratio as high as 6.2.[4] Treatment for AN is challenging as most patients lack insight into their illness and are reluctant to undergo weight restoration.

Multiple pieces of evidence suggest a strong genetic component in susceptibility to AN. A high degree of familial aggregation is observed for AN, with a first-degree relative recurrence risk >10.[5,6] An increased rate of AN is also seen among first-degree relatives of bulimia nervosa probands, and vice versa.[5] Twin studies have estimated AN heritability as ranging from 58 to 74% in two studies conducted in the United States,[7] 56% in a study conducted in Sweden[7] and 75% in a study conducted in Denmark.[8] The slight variability probably relates to differences in culture or ascertainment/diagnostic criteria.

Correspondence: Dr H Hakonarson, Department of Pediatrics, University of Pennsylvania, Philadelphia, PA 19104, USA. E-mail: hakonarson@email.chop.edu or wadeb@mail.med.upenn.edu
[7]See Appendix.
Received 5 April 2010; revised 3 August 2010; accepted 22 September 2010; published online 16 November 2010

Several studies have been conducted to investigate the genetic basis of AN.[9,10] A microsatellite linkage study mapped a locus on chromosome 1, which may harbor a risk allele for the most narrow diagnostic category of AN, the restricting-type AN (RAN).[11] single-nucleotide polymorphisms (SNPs) genotyping in this region[12] boosted the linkage signal and nominated susceptibility alleles of small effect, in two plausible candidate genes, namely *OPRD1* (delta opioid receptor) and *HTR1D* (serotonin 1D receptor). The association signals on these candidate genes were later confirmed by an independent study.[13] Linkage analyses using covariates, such as obsession-scale scores, identified multiple other genomic regions, which might harbor AN susceptibility alleles.[14,15] Several association studies on AN using candidate genes in selected pathways have also been conducted. In addition to studies on *HTR1D* and *OPRD1*, a study tested an SNP in the serotonin 2A receptor gene, but failed to find association.[16] A few other studies have tested genes in the dopamine pathway, and detected evidence for association for *DRD2* (dopamine D2),[17] but not for *COMT* (catechol-*O*-methyltransferase).[18] Positive evidence of association was also reported for *BDNF* (brain-derived neurotrophic factor),[19,20] but many other candidate gene association studies have generally failed to find significant association.[21] More recently, a Japanese group conducted genome-wide investigation of AN by typing pooled DNA on microsatellite markers with follow-up SNP genotyping, and detected significant association on rs2048332 near *SPATA17* on 1q41.[22] In summary, despite many linkage studies, candidate gene association studies and small-scale genome-wide association studies, the genetic architecture underlying AN susceptibility remains largely unknown.

To identify AN susceptibility loci, we performed a genome-wide association study (GWAS) using high-density SNP arrays on a large AN cohort. Given the known contribution of copy number variations (CNVs) to neuropsychiatric disorders,[23] we also performed a parallel study on CNVs, by taking advantage of the signal intensity data from SNP genotyping arrays. Here, we report the results from the analysis on both common SNPs and rare CNVs. Our study confirmed the potential role of known susceptibility genes, but also pointed to a few additional susceptibility genes and variants worthy of follow-up studies.

Materials and methods

Sample collection

The AN cases examined in this study were collected from several sources. A collaborative group to study AN genetics was formed ~15 years ago, including multiple AN clinical phenotypic experts.[24] Methods, ascertainment sites and diagnostic instruments for this effort have been previously described.[24] All cases met a lifetime diagnosis of DSM-IV AN, with or without amenorrhea, at least 3 years before study entry and by age 45. The amenorrhea criterion was waived because of its unreliability in retrospective assessment in females; data suggest that anorectic women with and without amenorrhea do not differ significantly.[25] The threshold for low weight was defined as a body mass index at or below 18 kg m^{-2}, which corresponds to the fifth percentile body mass index values of the National Health and Nutrition Examination Survey epidemiological sample of females[26] for the average age range (27–29 years) of the probands. Eating disorder symptoms and course of illness were assessed with the structured interview for anorexic and bulimic disorders.[27] Psychiatric comorbid disorders were assessed with semi-structured interviews and diagnoses were by DSM-IV criteria, using consensus best-estimate diagnostic procedures. Attitudes toward eating were quantified with the eating disorders inventory-2,[28] whereas measures of the severity of obsessions and compulsions were quantified with the Yale–Brown Obsessive-Compulsive Scale.[29] Personality constructs were measured by the temperament and character inventory,[30] whereas perfectionism was assessed by the Multi-dimensional Perfectionism Scale.[31] Potential probands were excluded if they had a history of severe CNS trauma, psychotic disorder or developmental disability, or if they had a medical or neurological condition that could confound the diagnosis of AN (such as type 1 diabetes). In total, we genotyped 1105 unrelated AN cases in this study, with the majority being female subjects. With stringent quality control measures (see below), a total of 1033 AN cases (1009 female subjects and 24 male subjects) were left in the GWAS analysis, including 39 without subtype classification. The distribution of the age of cases was 26.9 ± 8.6 years (mean ± s.d.), whereas the age at first symptom was 15.1 ± 3.1 years. The distribution of the lowest body mass index measure was 14.1 ± 2.0 kg m^{-2}, and the Yale–Brown Obsessive-Compulsive Scale was 15.1 ± 12.4.The disease subtypes were described in Supplementary Table 1.

The pediatric control group was recruited by the Children's Hospital of Philadelphia (CHOP) clinicians, nursing and medical assistant staff within the CHOP Health Care Network, which includes multiple primary care clinics and outpatient practices. The control subjects have linked electronic medical records in the clinical databases, and a coded version of these records can be accessed by researchers for genetic research purposes. At the time of the data analysis, the average age of the control subjects was 12.75 years with a s.d. of 4.2 years. One concern on using pediatric controls is the potential loss of power that is associated with the inability to exclude latent diagnoses of the phenotype of interest through intensive screening of controls; however, such loss of power is minimal when disease prevalence is below 1%,[32] so our study is unlikely to be significantly affected by this design. We utilized multi-dimensional scaling (MDS) on both AN cases and control subjects, together with 11 HapMap3 popula-

tions, to ensure good matching of genetic backgrounds (Supplementary Figure 1). Only the subsets of control subjects with genetically inferred European ancestry were used in the subsequent association analysis (Supplementary Figure 2). All these control subjects were genotyped using the same Illumina Human610 SNP arrays (Illumina, San Diego, CA, USA) at the same genotyping facility by the same technical staff as the case subjects. To further address the concern of population stratification, we also performed association analysis by logistic regression using principal components as covariates, based on the MDS analysis of cases and control subjects of European ancestry. The results were still similar without loci reaching genome-wide significance, and the genomic control inflation factor remained at 1.08. These control subjects genotyped on Human610 arrays were not used in previous publications. The Research Ethics Board of CHOP and other participating centers approved the study, and written informed consent was obtained from all subjects or their parents.

SNP genotyping and quality control

All DNA samples were genotyped on the Illumina Human610-Quad version 1 SNP arrays with ~610 000 markers (including ~20 000 non-polymorphic markers), at the Center for Applied Genomics, CHOP. Standard data normalization procedures and canonical genotype clustering files provided by Illumina were used to process the genotyping signals.

Following genotyping, we removed samples with >5% missing genotypes, after excluding ~20 000 non-polymorphic markers as they do not have genotypes. We used the PLINK software version 1.06[33] for MDS on markers not in linkage disequilibrium (LD) to identify the ancestry origin (via the --mds-plot argument), and removed samples who were not of genetically inferred European ancestry. Furthermore, based on the whole-genome identity-by-descent estimate, we eliminated samples from pairs of samples showing cryptic relatedness (identity-by-descent score > 0.2). A total of 1033 AN cases and 3733 control subjects were used in subsequent association tests on SNPs.

Association tests

For the genome-wide association analysis for SNPs, we utilized the PLINK software[33] version 1.06, through Cochran–Armitage trend test. For chromosome X markers, the analysis was performed on female subjects only. Additionally, we explored association analysis on the subset of female subjects only. Furthermore, given the previous reports on the linkage signals specifically for the cases with RAN,[11] we also examined association signals by performing the analysis on the subsets of RAN cases and control subjects. Additionally, we also explored restricting the analysis on AN cases with known family history, or AN cases with an age of onset before or equal to 16 years. We set a genome-wide significance threshold as $P < 1 \times 10^{-8}$, considering the multiple types of association tests that have been examined.

CNV calling and analysis

The Log R ratio (LRR) and B allele frequency (BAF) measures for all markers for all samples were directly calculated and exported from the Illumina BeadStudio software. The CNV calls were generated using the PennCNV software (version 2009 Aug27),[34] which utilizes an integrated hidden Markov model that incorporates multiple sources of information, including total signal intensity and allelic intensity ratio at each SNP marker, the distance between neighboring SNPs, the allele frequency of SNPs and the pedigree information where available. The default program parameters, library files and the genomic wave adjustment routine[35] in detect_cnv.pl program were used in generating CNV calls. The scan_region.pl program in PennCNV was used to map called CNVs to specific genes and exons, using the RefSeq gene definitions. The genomic coordinates for the CNV calls were generated using the NCBI build 36 human genome assembly.

Several procedures were used for quality control on the CNV calls. We first excluded all samples with LRR_standard deviation (SD) measure >0.3, or with BAF_drift measure >0.01, or with number of CNV calls >75. Next, we excluded sparse CNV calls, that is, those CNV calls with average inter-marker distance >50 kb (the average distance is ~5 kb across the whole genome for the arrays that we used). Furthermore, we excluded all CNV calls whose >50% genomic span overlap with known immunoglobulin regions (chr22: 20715572–21595082, chr14:105065301–106352275, chr2: 88937989–89411302, chr14:21159897–22090937), as these CNVs may be a result of somatic changes. In addition, we excluded CNV calls whose >50% genomic span overlap with centromere (a list of genomic coordinates for centromere in human genome NCBI 36 build were given at the PennCNV website FAQ section). The final set of CNV calls were then used in the comparative analysis between AN cases and control subjects. Large CNV calls that were potentially interesting were each visually confirmed using a custom script in PennCNV that generates JPG image files for CNVs.

Results

Genome-wide association of SNP data

We performed a GWAS in 1033 AN cases and 3773 pediatric control subjects, all of whom were genotyped by the Illumina Human610 SNP array with over 598 000 SNP markers. We utilized MDS analysis on a subset of SNP markers not in LD, to ensure that only samples with genetically inferred European ancestry were used in association tests (Supplementary Figure 1) and to ensure that cases and control subjects were well matched in their genetic ancestry (Supplementary Figure 2). The Manhattan plot for the whole-genome association tests was given in Figure 1.

None of the SNPs reached a stringent level of genome-wide significance, and the most significant marker is rs6959888 within *ZNF804B* on 7q21, with a *P*-value of 1.6×10^{-6} (Table 1). Its paralog *ZNF804A*

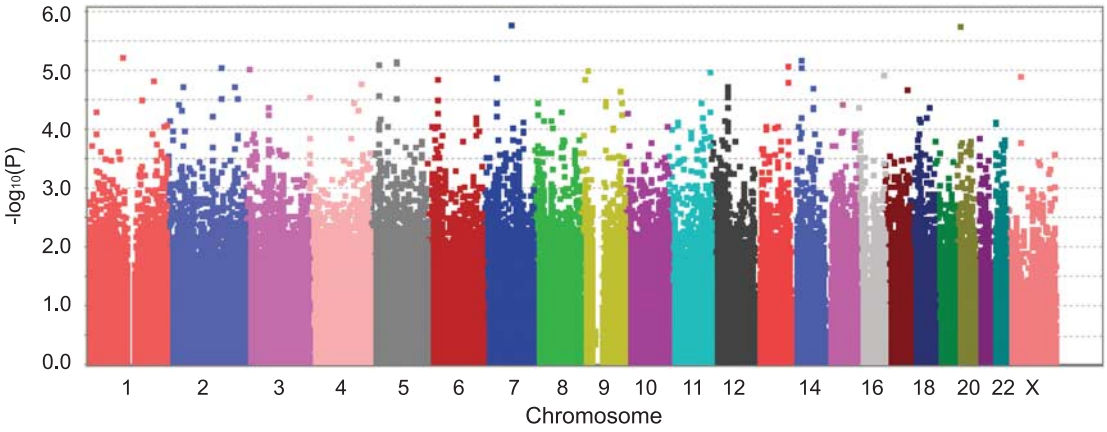

Figure 1 The Manhattan plot of logarithm of *P*-values vs genomic coordinates for whole-genome single-nucleotide polymorphism (SNP) markers. GWAS, genome-wide association study.

Table 1 The list of SNP markers that are most significantly associated with AN ($P<1\times10^{-5}$)

Index SNP[a]	*Locus*	*Closest gene*	*SNP-gene distance*	*MAF (case)*	*MAF (control)*	P-*value*	# *additional SNP*[b]
rs6959888	7q21.13	*ZNF804B*	0	0.15	0.11	1.63E-06	0
rs17725255	20p11.23	*CSRP2BP*	39396	0.14	0.11	1.72E-06	0
rs10494067	1p13.3	*NTNG1*	0	0.03	0.06	5.83E-06	0
rs2383378	14q12	*AKAP6*	0	0.35	0.41	6.41E-06	2
rs410644	5q14.1	*SSBP2*	40462	0.41	0.47	6.74E-06	2
rs4479806	5p14.1	*CDH9*	156926	0.06	0.10	7.79E-06	0
rs957788	13q33.3	*FAM155A*	0	0.37	0.31	8.11E-06	4
rs830998	2q31.1	*LRP2*	0	0.23	0.19	8.68E-06	3
rs6782029	3p25.3	*VGLL4*	0	0.19	0.24	9.04E-06	0
rs512089	9p21.3	*ELAVL2*	47984	0.28	0.24	9.85E-06	1
rs3808986	11q24.3	*APLP2*	29217	0.05	0.08	9.92E-06	0

AN, anorexia nervosa; MAF, minor allele frequency; SNP, single-nucleotide polymorphism.
[a]The most significantly associated SNP at each locus.
[b]The number of additional associated SNPs ($P<0.01$, $r^2>0.5$ with index SNP) at each locus.

was previously associated with schizophrenia,[36] but the function for neither gene has been characterized. The list of 10 additional most associated loci with $P<1\times10^{-6}$ were annotated in Table 1. Of note, several loci, including *APAK6*, *SSBP2*, *FAM155A*, *LPP2* and *ELAVL2*, harbor multiple SNPs with nominal significance levels. Performing logistic regression-based association tests incorporating principal components as covariates had minor effects on the results (Supplementary Table 2 and 3). For each of the most significant SNPs, we also examined the association statistics in (1) 1009 female cases and 1731 female control subjects (Supplementary Table 4); (2) 394 cases with RAN and 3773 control subjects (Supplementary Table 5); (3) 368 cases with a family history of eating disorders and 3773 control subjects (Supplementary Table 6) and (4) 489 cases with age of diagnosis $\leqslant$16 years and 3773 control subjects (Supplementary Table 7). Considering the several types of association tests that we have performed, appropriate genome-wide significance threshold should be regarded as $P<1\times10^{-8}$. When examining female subjects only, most of the SNPs showed decreased significance, except an SNP located within the intergenic region between *CDH10* and *CDH9* ($P=2.5\times10^{-8}$). The region was previously associated with autism spectrum disorders,[37] but the autism-associated SNP rs4307059 does not show evidence of association with AN ($P=0.45$). When comparing subjects with RAN and control subjects, none of the loci showed stronger evidence of association. When comparing cases with family history and control subjects, none of the loci showed stronger evidence of association. Similarly, when comparing subjects with early onset diagnosis and control subjects, we do not observe stronger evidence of association either. In summary, the list of loci in Table 1 did not reveal obvious candidate genes, but they represented a prioritized list of genes worthy of follow-up studies in additional independent cohorts.

Examination of previously reported candidate variants
We next examined whether we can confirm specific variants that were associated with AN in previously

published studies. Two independent studies have shown that two variants located in *OPRD1* and *HTR1D* on the 1p33–36 linkage region were associated with AN.[12,13] In the recent Brown *et al*.[13] study, rs569356 within *OPRD1* showed association with AN and RAN, whereas rs856510 and rs674386 ($r^2 = 1$ with each other) within *HTR1D* showed weak evidence of association with RAN only. In our study, we examined the SNP rs533123 within *OPRD1* ($r^2 = 0.95$ with rs569356 and 4 kb away), and confirmed its association and direction of effects with AN ($P = 1.5 \times 10^{-3}$, odds ratio = 1.2, minor allele frequency (MAF) = 21.7% in cases, MAF = 18.6% in controls). We also examined rs7532266 near *HTR1D* ($r^2 = 1$ with rs674386 and 30 kb away), but did not find evidence of association ($P = 0.44$, MAF = 31.1% in cases, MAF = 32.0% in controls). As the Brown *et al*. study showed association between *HTR1D* and RAN, but not with the broader diagnosis of AN, we also compared the subset of RAN cases and control subjects. We detected weak association signals for rs7532266 ($P = 0.04$, MAF = 28.5% in RAN cases) with the allelic effects observed in the same direction as previous reports.[13] There is no sample overlap between the Brown *et al*. and our study, and combining these studies by Fisher's combined method showed that *OPRD1* is associated with AN ($P = 1.76 \times 10^{-5}$), yet *HTR1D* is specifically associated with RAN ($P = 0.006$).

We also examined the SNP rs2048332 on 1q41 reported in a Japanese study.[22] This SNP was not placed on the Illumina Human610 array, so we examined rs6604568 instead, which has $r^2 = 0.84$ with rs2048332 and is only 15.6 kb away. The SNP rs6604568 was not associated with AN in our study ($P = 0.13$, MAF = 28.0% in cases, MAF = 29.7% in controls). Comparing RAN cases and control subjects did not reveal any association either ($P = 0.62$), suggesting that the previously reported association signals may be ethnicity specific. Furthermore, a CAG repeat polymorphism in *KCNN3* has been associated with AN.[38,39] The CAG repeat was not annotated in HapMap samples, so we examined all SNPs within *KCNN3*. We identified 85 SNPs within *KCNN3* and 6 SNPs near *KCNN3* (0.3–27 kb away), with the most significant SNP being rs906281 ($P = 0.002$). However, the SNPs may not tag the CAG repeat polymorphism.

Genome-wide investigation of CNVs

Besides common SNPs, rare CNVs have been associated with multiple related neuropsychiatric disorders, including schizophrenia,[40,41] autism[42] and bipolar disorder.[43] Thus, we next examined the role of rare CNVs in predisposing to AN. Using signal intensity data from SNP arrays, we generated CNV calls for 1015 AN cases and 3532 control subjects who passed quality control measures by PennCNV.[34] As increased 'CNV load' has been reported in schizophrenia cases,[40] we first examined whether we can find more CNVs in AN cases than control subjects. We caution that these types of 'load' analysis may be highly susceptible to data biases caused by signal quality in genotyping experiments, so we investigated several different thresholds for CNV calling. When utilizing default PennCNV parameters for CNV calling ($\geqslant 3$ markers), we observed slightly fewer CNVs in cases than controls (30.4 vs 31.2 per subject). When restricting the analysis to a set of more confident CNV calls ($\geqslant 10$ markers, CNV $\geqslant 100$ kb), we observed a similar number of CNVs in cases vs control subjects (3.0 vs 3.2 per subject). When examining large CNVs ($\geqslant 10$ markers, CNV $\geqslant 1$ Mb), similar observations were made (0.12 vs 0.12 per subject). No CNVs in cases or control subjects were > 2 Mb.

Given the similarity of total CNV loads in AN cases and control subjects, we next assessed whether AN cases tend to have more rare CNVs. For this analysis, we defined common CNV regions as those regions disrupted in $> 1\%$ subjects, and then classified CNVs as 'common' if $> 50\%$ of their genomic span overlap with a common CNV region, or 'rare' otherwise. In AN cases and control subjects, 22.7 and 22.7% of the CNVs were classified as rare CNVs, respectively. When examining rare CNVs > 100 kb, > 500 kb or > 1 Mb, we still could not obtain evidence that rare CNVs were over-represented in AN cases compared with control subjects. Therefore, the 'genetic load' of rare CNVs may have a less important function in AN susceptibility than in other psychiatric disorders.

Rare CNVs in AN cases point to specific regions and genes

As no CNV studies on AN have been published, we do not have any specific CNV findings to confirm. Instead, we first focused on a few CNVs implicated in other neuropsychiatric diseases such as schizophrenia, autism and epilepsy.[40,44–48] These include recurrent deletions/duplications on 1q21.1, 15q11.2, 15q13.3, 16p11.2, 16p13.1 and 22q11.2 as well as > 100 kb exonic deletions on *NRXN1*, all of which are very large and can be readily detected by the current SNP arrays. Given the small sample size, we did not expect to detect an association, and, therefore, presented descriptive results. In AN cases, we detected one 1.5 Mb deletion and one duplication on 1q21.1, three 370 kb deletions and 13 duplications on 15q11.2, one 800 kb deletion and one duplication on 16p13.1, as well as one 530 kb duplication on 16p11.2. These individuals are all female. Clinically, they do not appear to stand out relative to other cases with the exception that the case with the 1q21.1 deletion reported a lifetime minimum body mass index of 11.4, which is extremely low, even for individuals with AN. In comparison, in control subjects, we observed 16 deletions and 21 duplications on 15q11.2, one deletion on 15q13.3, two deletions and eight duplications on 16p13.1, one deletion and three duplications on 16p11.2. Although the 15q11.2 duplication showed some evidence of association ($P = 0.036$, two-sided Fisher's exact test), it did not pass adjustment for multiple testing. These

Table 2 A list of rare and large (>500 kb, <1%) CNVs observed in AN cases

Region	*#SNP*	*Length*	*Type*	*Genes*
chr1:104048591–104582290	81	533 700	Del	*AMY1A,AMY1B,AMY1C*
chr1:185348953–185890594	119	541 642	Del	*(intergenic)*
chr1:187261219–188096756	175	835 538	Dup	*(intergenic)*
chr1:73150625–74188548	132	1 037 924	Dup	*(intergenic)*
chr10:18928375–19460187	123	531 813	Del	*ARL5B,NSUN6*
chr10:36911902–37727526	170	815 625	Dup	*ANKRD30A*
chr10:56377654–57417982	223	1 040 329	Del	*(intergenic)*
chr11:48364239–48915263	88	551 025	Del	*OR4A47*
chr11:50021328–50687058	71	665 731	Dup	*LOC441601,LOC646813*
chr11:50052490–50687058	68	634 569	Dup	*LOC441601,LOC646813*
chr12:128372933–129037385	280	664 453	Dup	*TMEM132D*
chr12:36301572–36995204	68	693 633	Dup	*(intergenic)*
chr12:42158820–43041461	143	882 642	Dup	*ADAMTS20,IRAK4,PUS7L,TMEM117,TWF1*
chr13:22426685–23795901	510	1 369 217	Del	*C1QTNF9,C1QTNF9B,MIPEP,MIR2276,PCOTH,SACS,SGCG,SPATA13,TNFRSF19*
chr13:22426685–23813547	513	1 386 863	Del	*C1QTNF9,C1QTNF9B,MIPEP,MIR2276,PCOTH,SACS,SGCG,SPATA13,TNFRSF19*
chr13:53976442–54662634	129	686 193	Del	*(intergenic)*
chr14:42999532–43775591	165	776 060	Del	*(intergenic)*
chr15:18822301–20049770	122	1 227 470	Dup	*BCL8,CXADRP2,GOLGA6L6,GOLGA8C,LOC646214, LOC727924,NF1P1,OR4M2,OR4N3P,OR4N4,POTEB*
chr16:78285281–78944520	250	659 240	Dup	*(intergenic)*
chr16:78847246–79415644	213	568 399	Dup	*CDYL2,DYNLRB2,MIR548H4*
chr17:10031526–10593105	120	561 580	Dup	*C17orf48,GAS7,MYH1,MYH13,MYH2,MYH3,MYH4,MYH8,SCO1,TMEM220*
chr17:69348935–69890764	147	541 830	Dup	*BTBD17,DNAI2,GPR142,KIF19,MGC16275,RPL38,TTYH2*
chr17:69561788–70081239	132	519 452	Dup	*BTBD17,CD300A,CD300C,CD300LB,DNAI2,GPR142, GPRC5C,KIF19,MGC16275,RPL38,TTYH2*
chr2:193776979–195000115	96	1 223 137	Dup	*(intergenic)*
chr2:32487194–33181898	154	694 705	Dup	*BIRC6,LOC100271832,LOC285045,LTBP1,MIR558,TTC27*
chr2:3778371–4619354	212	840 984	Del	*(intergenic)*
chr22:47785528–48365092	277	579 565	Dup	*(intergenic)*
chr3:1193279–2145519	446	952 241	Dup	*CNTN4,CNTN6*
chr3:1307197–2183832	388	876 636	Del	*CNTN4,CNTN6*
chr3:59702842–60998395	642	1 295 554	Dup	*FHIT*
chr3:81164179–82956294	249	1 792 116	Dup	*GBE1*
chr3:832325–1403635	260	571 311	Dup	*CNTN6*
chr4:181763921–182314469	116	550 549	Del	*(intergenic)*
chr4:186249595–186808435	140	558 841	Dup	*ANKRD37,C4orf47,CCDC110,KIAA1430,LRP2BP,PDLIM3, SLC25A4,SNX25,SORBS2,UFSP2*
chr4:190385789–190993476	114	607 688	Dup	*(intergenic)*
chr5:109203066–110445774	232	1 242 709	Del	*MAN2A1,MIR548C,SLC25A46,TMEM232,TSLP*
chr5:69199857–69713288	83	513 432	Del	*LOC100170939,LOC653391,SERF1A,SERF1B,SMN1,SMN2*
chr5:84799217–85510893	126	711 677	Dup	*(intergenic)*
chr5:8482773–9257142	219	774 370	Dup	*SEMA5A*
chr6:164618476–165431910	193	813 435	Dup	*(intergenic)*
chr6:75980325–76529791	68	549 467	Dup	*COX7A2,FILIP1,MYO6,SENP6,TMEM30A*
chr7:108490386–110069263	304	1 578 878	Del	*EIF3IP1*
chr7:124752465–125581531	151	829 067	Del	*(intergenic)*
chr7:153093739–153705030	134	611 292	Dup	*DPP6*
chr7:88149364–88779880	178	630 517	Dup	*MGC26647,ZNF804B*
chr9:10743219–11270652	132	527 434	Del	*(intergenic)*
chr9:28113402–29308695	354	1 195 294	Del	*LINGO2,MIR873,MIR876*

Abbreviations: AN, anorexia nervosa; CNV, copy number variation; Del, deletions; Dup, duplications; SNP, single-nucleotide polymorphism.

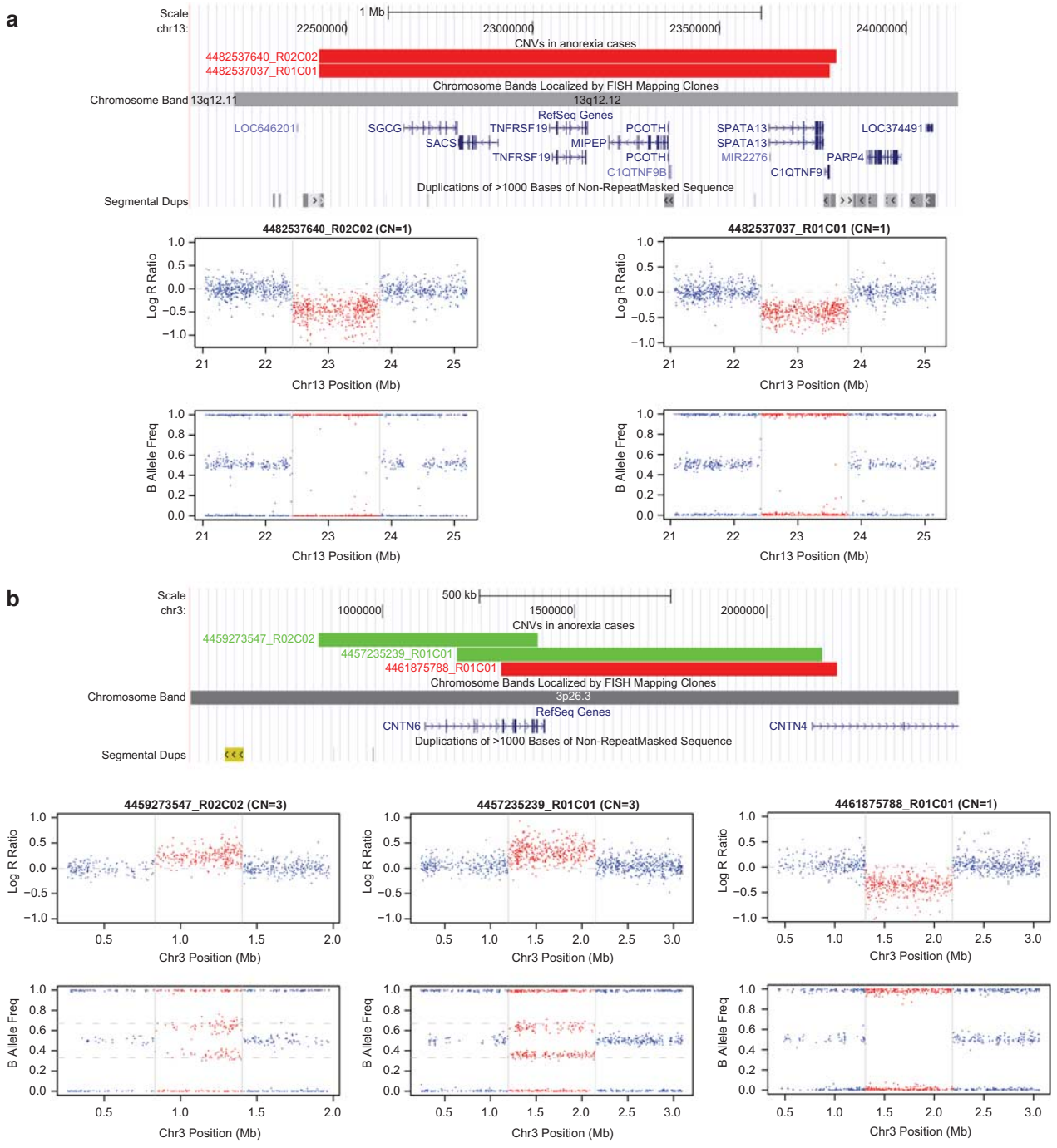

Figure 2 Genome Browser shots and visual validation on (**a**) 13q12 deletions and (**b**) deletions/duplications disrupting the *CNTN4/CNTN6* region. The predicted copy number variation (CNV) region was enclosed within two gray vertical lines, whereas the red dots represent markers within the CNV. The Log R ratio (LRR) plot shows total signal intensity, and decreased or increased LRR suggests the presence of deletions or duplications. The B allele frequency (BAF) plot shows allelic intensity ratio, and lack of BAF around 0.5 suggests the presence of deletions, yet BAF clusters around 0.33 and 0.66 suggest the presence of duplications.

results were also summarized in Supplementary Table 8.

We next examined rare and large CNVs in AN cases to determine whether some of them tended to cluster in the same genomic region (Table 2). Interestingly, we identified a large and rare CNV on 13q12 (chr13:22426685–23795901, ~1.4 Mb) that was present in two AN cases (Figure 2a). The CNVs were not detected in control subjects in our study. To identify the true population frequency of this particular CNV, we subsequently examined a larger data set comprising over 72 918 subjects genotyped by the Illumina

550K or 610K arrays (all samples were genotyped at the CHOP), and identified 38 subjects carrying the 13q12 CNV (>80% base overlap), including 15 deletions and 23 duplications. Therefore, the population frequency of this deletion is ~1/5000 vs 1/500 for AN; however, this 10-fold enrichment could be inflated by winner's curse, and we note that the 95% confidence interval for odds ratio is 1.06–41.3. Several genes were enclosed in this CNV; among them, *SCAS* is highly expressed in neuronal tissues, and mutations in this gene result in autosomal recessive spastic ataxia.[49] Additionally, one duplication (877 kb) and one deletion (952 kb) both disrupt the *CNTN6* and *CNTN4* genes and their intergenic regions (Figure 2b). Deletions or translocations at this region have been reported in autism, developmental delay and other diseases.[50,51] Considering that the case:control ratio in our data set is not 1:1, in addition to the analysis on case-specific CNVs, we also listed all large and rare (>500 kb, <1%) CNVs in Table 2. Given the relatively small sample size, none of these regions would reach statistical significance. However, rare genetic variations with high penetrance may point to important candidate genes that are worthy of follow-up analysis.

Discussion

In this study, we performed genome-wide investigation of both SNPs and CNVs to identify genetic variants associated with AN. To our knowledge, this is the first study that utilized whole-genome and high-density genotyping technology to investigate the genetic basis of AN, and our analysis identified specific genes and regions that are worthy of additional studies in independent cohorts. However, while the CNV loci identified are compelling, we also wish to discuss several caveats related to the study design and the interpretation of the results.

First, despite the relatively large sample size (>1000 cases) compared with any previous association studies on AN, we were still unable to find genome-wide significant signals. This observation is similar to previous modest-size studies on bipolar disorder and schizophrenia, and may suggest that much larger sample sizes are needed to identify common variants with small effects for neuropsychiatric disorders. Alternatively, it may also suggest that rare variants probably have more significant functions for AN, and are hence difficult to detect without much larger sample sizes in GWAS. This observation motivates us to supplement common SNP analysis with rare CNV analysis. However, we were unable to garner support that rare CNVs tend to be enriched in cases compared with control subjects. While acknowledging that such 'genetic load' analysis is highly susceptible to noises in the data and differential biases in genotyping experiments, these results seem to suggest that rare variants, including recurrent CNVs and non-recurrent ones, appear to have a less important function, compared with other psychiatric illness such as schizophrenia and autism.[40,41,44,45]

Second, compared with SNPs, given the rare nature of large CNVs that disrupt genes, it requires even larger sample sizes to establish the true association of CNVs and disease phenotypes. Therefore, although the genes identified by our rare CNV analysis (Table 2) are intriguing candidates, additional sample sets with whole-genome genotyping data are necessary to further validate these results and establish confirmatory associations. On the other hand, in this study, we did not specifically test for common CNVs, in part because common CNVs tend to be small (with increased false negative rates to detect by computer algorithms), and they are already well tagged by the SNP genotyping arrays.[52]

Finally, given that the phenotypic presentation of AN could differ between various subtypes, we have also performed subgroup-based analysis on multiple subgroups, including RAN, AN cases with family history, or AN cases with early age of diagnosis. However, none of these subtype analysis yielded genome-wide significant results. We note that this analysis further reduced sample size, so it has even smaller power to detect true associations. Nevertheless, it would be important and reasonable to explore whether specific association signals tend to be stronger or even tend to exist exclusively in specific subtypes of the diseases. For example, in our study, the *OPRD1* association with AN was observed exclusively in the comparison between RAN and control subjects, and no evidence of association was found for the entire AN group.

We also find it important to discuss our results in comparison with other neuropsychiatric/neurodevelopmental diseases such as schizophrenia, bipolar disorder and autism spectrum disorders. Unlike other psychiatric disorders, the clinical diagnosis of AN is relatively straightforward, and it is much less likely that an incorrect diagnosis is given to AN cases. Additionally, the phenotypes are much less heterogeneous than affective disorders or autism spectrum disorders. Therefore, one would assume that phenotypic heterogeneity is less of a concern for AN. On the other hand, there are several unique challenges for the genetic analysis of AN. Compared with other psychiatric disorders, AN may be more likely to be related to cultural differences, which may complicate the genetic studies. The relative contributions of culture and biology to AN have been long debated in this field; future association analyses that incorporate cultural background or socioeconomic status may lead to improved power and precision to identify AN genes.

In conclusion, through genome-wide association analysis of both SNPs and CNVs, we have confirmed two previously published candidate genes, yet also identified additional genes that are likely to be involved in AN pathogenesis. These genes require testing in additional independent data sets. The study illustrates the importance of using whole-genome and

unbiased genomic survey to identify disease susceptibility genes and mechanisms.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We gratefully thank all the patients and their families who were enrolled in this study, as well as all the control subjects who donated blood samples to Children's Hospital of Philadelphia (CHOP) for genetic research purposes. We thank the Price Foundation for their support of the Collaborative Group effort that was responsible for recruitment of patients, collection of clinical information and provision of the DNA samples used in this study. The authors also thank the Klarman Family Foundation for supporting the study. We thank the technical staff at the Center for Applied Genomics at CHOP for producing the genotypes used for analyses and the nursing, medical assistant and medical staff for their invaluable help with sample recruitments. CTB and NJS are funded in part by the Scripps Translational Sciences Institute Clinical Translational Science Award [Grant Number U54 RR0252204-01]. All genome-wide genotyping was funded by an Institute Development Award to the Center for Applied Genomics from the CHOP.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)

Appendix

Members of the Price Foundation Collaborative Group

Harry Brandt[7], Steve Crawford[7], Scott Crow[8], Manfred M Fichter[9], Katherine A Halmi[10], Craig Johnson[11], Allan S Kaplan[12,13,14], Maria La Via[15], James Mitchell[16,17], Michael Strober[18], Alessandro Rotondo[19], Janet Treasure[20], D Blake Woodside[13,14,21], Cynthia M Bulik[15,22], Pamela Keel[21], Kelly L Klump[23], Lisa Lilenfeld[24], Laura M Thornton[15], Kathy Plotnicov[25], Andrew W Bergen[26] and Pierre Magistretti[27]

[7]Department of Psychiatry, University of Maryland School of Medicine, Baltimore, MD, USA
[8]Department of Psychiatry, University of Minnesota, Minneapolis, MN, USA
[9]Roseneck Hospital for Behavioral Medicine, Prien, Germany and Department of Psychiatry, University of Munich (LMU), Munich, Germany
[10]New York Presbyterian Hospital-Westchester Division, Weill Medical College of Cornell, University, White Plains, NY, USA
[11]Eating Recovery Center, Denver, Colorado, USA
[12]Center for Addiction and Mental Health, Toronto, Canada
[13]Department of Psychiatry, Toronto General Hospital, University Health Network, Toronto, Canada
[14]Department of Psychiatry, University of Toronto, Toronto, Canada
[15]Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[16]Neuropsychiatric Research Institute, Fargo, ND, USA
[17]Department of Clinical Neuroscience, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND, USA
[18]Department of Psychiatry and Biobehavioral Sciences, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA
[19]Department of Psychiatry, Neurobiology, Pharmacology, and Biotechnology, University of Pisa, Pisa, Italy
[20]Dept Academic Psychiatry, Bermondsey Wing Guys Hospital, University of London, UK
[21]Department of Psychology, Florida State University, Tallahassee, FL, USA

[22]Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[23]Department of Psychology, Michigan State University, East Lansing, MI, USA
[24]Clinical Psychology Program, Argosy University, Washington, DC, USA
[25]Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA, USA
[26]Center for Health Sciences, SRI International, Menlo Park, CA, USA
[27]Brain Mind Institute, EPFLCH-1015 Lausanne, Switzerland