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REPORT

A Genome-wide Study Reveals Copy Number Variants Exclusive to Childhood Obesity Cases

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The prevalence of obesity in children and adults in the United States has increased dramatically over the past decade. Genomic copy number variations (CNVs) have been strongly implicated in subjects with extreme obesity and coexisting developmental delay. To complement these previous studies, we addressed CNVs in common childhood obesity by examining children with a BMI in the upper 5th percentile but excluding any subject greater than three standard deviations from the mean in order to reduce severe cases in the cohort. We performed a whole-genome CNV survey of our cohort of 1080 defined European American (EA) childhood obesity cases and 2500 lean controls (< 50th percentile BMI) who were genotyped with 550,000 SNP markers. Positive findings were evaluated in an independent African American (AA) cohort of 1479 childhood obesity cases and 1575 lean controls. We identified 17 CNV loci that were unique to at least three EA cases and were both previously unreported in the public domain and validated via quantitative PCR. Eight of these loci (47.1%) also replicated exclusively in AA cases (six deletions and two duplications). Replicated deletion loci consisted of *EDIL3*, *S1PR5*, *FOXP2*, *TBCA*, *ABCB5*, and *ZPLD1*, whereas replicated duplication loci consisted of *KIF2B* and *ARL15*. We also observed evidence for a deletion at the *EPHA6-UNQ6114* locus when the AA cohort was investigated as a discovery set. Although these variants may be individually rare, our results indicate that CNVs contribute to the genetic susceptibility of common childhood obesity in subjects of both European and African ancestry.

Obesity (MIM 601665) is a major health problem in modern societies, with increasing prevalence in Western societies, particularly in children.[1] Obesity and its associated phenotype, insulin resistance, is also considered a contributor to the major causes of death in the United States and is an important risk factor for type 2 diabetes (T2D [MIM 125853]), cardiovascular diseases (CVD), hypertension (MIM 145500), and other chronic diseases. Approximately 70% of obese adolescents grow up to become obese adults;[2] indeed, obesity present in adolescence has been shown to be associated with increased overall mortality in adults.[3]

Despite environmental changes over the last 30 years, in particular the unlimited supply of convenient, highly calorific foods together with a sedentary lifestyle, there is also strong evidence for a genetic component to the risk of obesity.[4,5] This is reflected in prevalence differences between racial groups. In addition, the familial occurrences of obesity have been long noted with the concordance for fat mass among monozygotic twins, reported to be 70%–90%, higher than the 35%–45% concordance in dizygotic twins; as such, the estimated heritability of BMI ranges from 30% to 70%.[6–8]

In the past three years, 13 genetic loci have been implicated in BMI from the outcomes of genome-wide association studies (GWAS), primarily in adults. Insulin-induced gene 2 (*INSIG2* [MIM 608660]) was the first locus to be reported by this method as having a role in obesity,[9] but replication attempts have yielded inconsistent outcomes. The second reported locus, the fat mass- and obesity-associated gene (*FTO* [MIM 610966]),[10] has been more robustly observed in other cohorts, including our own.[11–14] Subsequent larger studies have uncovered 11 additional loci;[15–17] in addition, copy number variation (CNV) studies of extreme obesity in children, half of which had developmental delay, reported rare variants contributing to the trait.[18,19]

In the current study we examined CNVs in a large pediatric cohort presenting with common obesity that is primarily nonsyndromic. For this purpose, we performed a whole-genome CNV study in a large cohort of childhood-obesity cases and lean controls of European ancestry who were genotyped with the Illumina Infinium II HumanHap550K BeadChip. The genotype data content together with the intensity data provided by the array

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provided high confidence for CNV calls. Importantly, the simultaneous analysis of intensity data and genotype data in the same experimental setting established a highly accurate definition for normal diploid states and any deviation thereof. To call CNVs, we used the PennCNV algorithm,[20] which combines multiple sources of information, including log R ratio (LRR) and B allele frequency (BAF) at each SNP marker, along with SNP spacing, a trained hidden Markov model, and population frequency of the B allele to generate CNV calls. Each sample had CNV calls blinded with respect to case status. Positive findings were evaluated in an independent replication cohort of similar size and of African ancestry. A flow diagram of the CNV filtering and testing steps is provided in Figure S1 (available online).

We calculated quality-control measures in our HumanHap550 GWAS data on the basis of statistical distributions to exclude poor-quality DNA samples and false-positive CNVs. The first threshold is the percentage of attempted SNPs that were successfully genotyped. Only samples with call rates $> 98\%$ and a standard deviation (SD) of LRR < 0.30 were included. Wave artifacts roughly correlating with GC content resulting from hybridization bias of low full-length DNA quantity are known to interfere with accurate inference of CNVs,[21] so only samples in which the GC wave factor of the LRR ranged between $-0.1 < X < 0.1$ were accepted. If the count of CNV calls made by PennCNV exceeded 100 (Figure S2), the DNA quality was considered poor; thus, only samples with a CNV call count < 100 were included. Any duplicate samples (such as monozygotic twins) had one sample excluded.

Our study consisted of 1080 European American (EA) obese children (BMI $\geq 95^{th}$ percentile), 2500 EA lean controls (BMI $< 50^{th}$ percentile), 1479 African American (AA) obese children, and 1575 AA lean controls who met strictly established data-quality thresholds for CNVs. All subjects were consecutively recruited from the Greater Philadelphia area from 2006 to 2009 at the Children's Hospital of Philadelphia, were biologically unrelated on the basis of available SNP data (pairwise identity by descent [IBD] was used to filter out individuals showing relatedness above 0.25), and were between 2 and 18 years old (Table S1 outlines the age and gender distribution in the obese and lean groups in the ethnicities separately). This study was approved by the institutional review board of the Children's Hospital of Philadelphia. Parental informed consent was given for each study participant for both the blood collection and the subsequent genotyping. Global ancestry was determined with the use of Eigenstrat to ensure a singular cluster of ethnic ancestry in the European and African populations. BMI $\geq 95^{th}$ percentile was defined with the use of the Center for Disease Control (CDC) Z-score $= 1.645$. However, all subjects had to be between -3 and $+3$ SD of the CDC-corrected BMI in order to exclude outliers that could potentially be a result of measurement error or to eliminate the majority of presumed syndromic cases in the cohort; this cutoff therefore provided us with a cohort consisting primarily of common obesity, in contrast to the severe obesity cases previously reported,[18,19] which included only cases with > 3 SD. We carefully reviewed the phenotypes of our controls to remove anyone with a syndrome, multisystemic disorder, or developmental delay.

An average of 22.0 and 19.5 CNV calls per individual were made with the PennCNV software in the EA cases and controls, respectively, 93% of subjects having 8–45 CNV calls (Figure S2). We called four different copy number states for the EA case and control cohorts, including 919 and 2109 homozygous deletions (copy number [CN] = 0), 13,678 and 28,917 hemizygous deletions (CN = 1), 6767 and 16,689 one-copy duplications (CN = 3), and 344 and 1139 two-copy duplications (CN = 4), respectively. Figure S3 shows an example of raw Illumina data as viewed in the BeadStudio software and the resulting CNV call. The CNV calls spanned from 3 to 7903 SNPs, with an average of 19 SNPs per CNV call, and their sizes ranged from 50 bp to 33 Mb, with an average size of 81 kb.

Ninety-three percent of AA subjects also harbored 8–45 CNV calls (Figure S2). An average of 24.1 and 25.1 CNV calls per individual were made in AA cases and controls, respectively. Among them, we identified 897 and 1026 (CN = 0), 25,650 and 28,793 (CN = 1), 8778 and 10,251 (CN = 3), and 418 and 535 (CN = 4) CNV calls, respectively. The CNV calls spanned from 3 to 7903 SNPs, with an average of 15 SNPs per CNV call, and their sizes ranged from 50 bp to 33 Mb, with an average size of 67 kb.

To identify genomic loci potentially contributing to common childhood obesity in the EA subjects, we applied a segment-based scoring approach that scans the genome for consecutive SNPs with more frequent copy number changes in cases compared to controls. The genomic span for these consecutive SNPs delineates common CNV regions (CNVRs).

We assessed local ancestry by using the 1 MB region surrounding each CNV locus, which included an average of 300 SNP genotypes, resulting in well-clustered populations without significantly deviating individuals. We report statistical local minimums to narrow the association in reference to a region of nominal significance, including SNPs residing within 1 Mb of each other. Resulting significant CNVRs were excluded if they met any of the following criteria: (1) residing on telomere or centromere proximal cytobands; (2) arising in a "peninsula" of common CNV arising from variation in boundary truncation of CNV calling; (3) located in genomic regions with extremes in GC content that produce hybridization bias; or (4) made up of samples contributing to multiple CNVRs.

We identified 34 putative CNVR loci (15 deletions and 19 duplications) that were exclusively present in at least three EA cases (Fisher's exact test $p \leq 0.05$); however, three of the deletions proved to be false positives during the validation process with quantitative PCR (qPCR), a method commonly used for independent validation of CNVs (Table S2 and Figure S4). All of our experimental validations were conducted in a blinded manner by an independent investigator group so as to minimize any bias, which

adds another level of confidence to the experimental validation results. Issues related to CNV recurrence, accuracy, locus dependence, and difficulties related to GC content were alleviated by running qPCR in quadruplicate, observing low SD across runs, assaying control samples for the same genomic region, and assaying the same samples in different genomic regions. This validation process involved probes being selected with the ProbeFinder v2.41 software (Roche, Indianapolis, IN, USA), and qPCR was then performed on an Applied Biosystems 7500 Real Time PCR Instrument or on an Applied Biosystems Prism 7900HT Sequence Detection System. Each sample was analyzed in quadruplicate, either in 25 μl reaction mixture (250 nM probe, 900 nM each primer, Fast Start TaqMan Probe Master from Roche, and 10 ng genomic DNA) or in 10 μl reaction mixture (100 nM probe, 200 nM each primer, 1× Platinum Quantitative PCR SuperMix-Uracil-DNA-Glycosylase [UDG] with ROX from Invitrogen, and 25 ng genomic DNA). The values were evaluated with Sequence Detection Software v2.2.1 supplied by the manufacturer. Data analysis was further performed via the $\Delta\Delta C_T$ method. Reference genes, chosen from *COBL* (MIM 610317), *GUSB* (MIM 611499), and *SNCA* (MIM 163890), were included on the basis of the minimal coefficient of variation, and data were then normalized by setting a normal control to a value of 1. None of these rare CNVs could be effectively tagged by a single common SNP present on the array (Table S3).

Only 17 of these CNVR loci were unique to our cohort, i.e., not reported in controls by the Database of Genomic Variants, of which eight (47.1%) also replicated exclusively in AA cases (six deletions and two duplications) (Table 1). The use of a different racial group for replication purposes represents a higher bar than making similar attempts in the same ethnicity. For example, when the common variant in *TCF7L2* (MIM 602228) associated with type 2 diabetes was also associated with the disease in Africans with a similar magnitude,[22] it was considered much more established as having a role in the disease; in addition, our recent asthma GWAS finding was also observed in African Americans, which gave us a much higher level of certainty of our observation.[23] Since many variants have been shown to have different frequencies in different ethnic groups, observation of the same CNV as exclusive in EA and AA cohorts separately for the same phenotype minimizes the potential for confounding effects of population stratification. Figure 1 and Figure 2 visualize representative loci most frequently harboring deletions or duplications, with the use of the UCSC Genome Browser, build hg18 (March 2006) of the human genome. We also evaluated whether any of the key CNVs cosegregated with obesity in families, where data was available, and we were able to establish that at least four of the CNVs were inherited from an obese parent and at least four were de novo (Table S4).

In order to further establish the significance of these findings, we looked at the converse situation, i.e., exclusive CNVRs among the controls. First, whereas exclusivity to three obese subjects constituted a nominally significant observation among EA cases, ten EA controls were required to reach the same significance threshold because of the abundance of controls in our study. Analyzed in this way, we observed no exclusive deletions and only four exclusive duplications in the EA controls; however, none of these duplications replicated exclusively in the AA controls. As such, in contrast to these control observations, we see a highly significant overabundance of exclusive CNVRs in our EA cases that go on to replicate exclusively in our AA cases, revealing that we have a $< 12\%$ false-positive discovery rate.

We also evaluated large rare deletions present in $< 1\%$ of individuals and > 500 kb in size, as set previously,[18] and we did not observe an excess of large rare deletions genome-wide (Table S5). This is not unexpected, given that the previous report found significance only when including developmental delay subjects and not when severe early-onset obesity was evaluated alone.

The loci harboring exclusive deletions in cases of both ethnicities consisted of four genes directly overlapping, namely *S1PR5* (endothelial differentiation, sphingolipid [MIM 605146]), *FOXP2* (forkhead box P2 [MIM 605317]), *TBCA* (tubulin-specific chaperone a [MIM 610058]) and *ABCB5* (ATP-binding cassette, sub-family B, member 5 [MIM 611785]), whereas two deletions were closest to *EDIL3* (EGF-like repeats- and discoidin I-like domains-containing protein 3 [MIM 606018]) and *ZPLD1* (zona pellucida-like domain containing 1). Pairwise IBD, both global and local, yielded low values close to zero among the five cases with the *EDIL3* deletion; although exact CNV breakpoint sharing between cases was not a necessary parameter of our analysis, it does suggest that a specific copy number polymorphism is observed. The two loci harboring exclusive duplications in cases from both ethnicities consisted of one gene directly overlapping, namely *ARL15* (ADP-ribosylation factor-like 15), and one closest to *KIF2B* (kinesin family member 2B).

The majority of genes residing at the loci uncovered in this study have not been previously implicated in obesity. However, the most notable finding is with *ARL15,* which was recently uncovered in a GWAS of adiponectin levels, with the same risk allele also being associated with a higher risk of coronary heart disease and type 2 diabetes.[24]

In addition, although members of the human Forkhead box (FOX) gene family have been strongly implicated in metabolic traits,[25,26] mutations in *FOXP2* are best known for causing developmental speech and language disorders in humans.[27] We thoroughly evaluated the questionnaires and medical records of the children harboring the *FOXP2* deletion and found that five do not have any speech problems; however, one child with the deletion has isolated speech delay, with no evidence of any underlying syndromes or intellectual impairment, and is receiving speech therapy at age 11. Indeed, poor speech, developmental delay, and obesity are commonly observed concomitantly in children with Prader-Willi syndrome.[28,29]

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Table 1. CNVs at the SNP Level Exclusive to Childhood Obesity in the European American Cohort, which Were Both Not Present in the Public Domain and qPCR Validated, Together with the Replication Attempt Data in African Americans

	EA	AA				
CNV	**No. of Cases Harboring Exclusively**	**SNP Tested**	**No. of Cases Harboring**	**No. of Controls Harboring**	**Distance from Nearest Gene (bp)**	**Nearest Gene**
Deletions						
chr5:83835179–83874339	***5***	***rs10051401***	***1***	***0***	***118812***	***EDIL3***
chr19:10489548–10512171	***3***	***rs11670254***	***5***	***0***	***0***	***S1PR5***
chr7:113843696–113859679	***3***	***rs12705964***	***3***	***0***	***0***	***FOXP2***
chr5:77039051–77076628	***3***	***rs384109***	***2***	***0***	***0***	***TBCA***
chr7:20708193–20711088	***3***	***rs12700232***	***1***	***0***	***0***	***ABCB5***
chr3:104059109–104092618	***3***	***rs1144781***	***1***	***0***	***377734***	***ZPLD1***
chr12:16141980–16149714	3	rs1376332	0	0	60398	*DERA*
chr10:118354808–118363319	3	rs7905887	0	1	0	*PNLIPRP1*
chr20:5111828–5137472	3	rs17785402	0	0	0	*BC015432-CDS2*
chr4:35782722–35828015	3	rs4833119	1	1	0	*CENTD1*
Duplications						
chr17:49444406–49449022	***3***	***rs17730346***	***2***	***0***	***186834***	***KIF2B***
chr5:53467427–53480255	***3***	***rs16882296***	***1***	***0***	***0***	***ARL15***
chr12:21446005–21459060	3	rs7953750	0	0	6367	*SLCO1A2*
chr6:67391945–67395314	3	rs183895	0	2	918106	*EGFL11*
chr5:90155080–90157263	3	rs7717221	0	0	0	*GPR98*
chr13:61409439–61413267	3	rs2183953	0	0	509359	*PCDH20*
chr2:21337060–21342588	3	rs870638	0	0	216610	*APOB*

EA, European Americans; AA, African Americans; CNV, copy number variant. Those CNVs that replicated exclusively in AA cases are indicated with bold and italics. Coordinates are derived from UCSC Genome Browser build hg18. The "Distance from Nearest Gene" column lists 0 if the CNV encompasses the gene listed or a value if the CNV is nearby the gene listed. These loci showed a copy number (CN) = 1 for deletion loci and a CN = 3 for duplication loci across contributing samples. No other states, such as CN = 0 or CN = 4, were observed at these loci. The individual CNV calls are assigned as a SNP-based statistic. Therefore, contiguous SNPs showing case nonzero values and control zero values form a region of copy number variation (CNVR), which are the CNVRs listed. The SNP tested is the central SNP in the CNVR so as to ensure exact overlap in replication

We elected to use the EA cohort for discovery because of the much larger control group available in this ethnicity to observe and establish exclusivity most comprehensively in the discovery setting. However, in order to explore the possibility of additional exclusive loci, we also went the other way; i.e., we analyzed the AA cohort as the discovery cohort and attempted to replicate in the EA cohort (Table S6). Although exclusivity to five AA cases was required to achieve nominal significance in the discovery stage in this setting because of the fewer lean controls in this cohort (Table S7), we did observe evidence for a deletion at the *EPHA6-UNQ6114* locus (MIM 600066) that was exclusive to AA cases.

To date, there has been a notable paucity of GWA studies in childhood obesity, with studies primarily uncovering loci in the adult setting,[10,12,14–17] and no study to date has reported CNVs that are significantly associated with the common obesity trait as opposed to the severe form.[18,19] As such, our study represents a large-scale, unbiased genome-wide scan of CNVs in common pediatric obesity. The loci uncovered in this present study are exclusively observed in childhood obesity and replicated in an independent case control data set from a different ethnicity. The results are given extra credibility because the well-established *FTO*[10–14] locus is very strongly associated with both BMI and obesity in our EA cohort.[11,30] We did not observe association with any CNVs previously reported in subjects with severe pediatric obesity and coexisting developmental delay.[18] In addition, we did not have probe coverage of the previously reported common CNV flanking *NEGR1* (MIM 613173) or encompassing *SH2B1* (MIM 608937).[17]

Because these variants are individually rare, we would postulate that they do not contribute greatly to the overall "missing heritability" in obesity, but they do present us with potential insights in to the underlying biology of childhood obesity; however, to the individuals harboring these variants, they are very relevant to their disorder. With new advances in sequencing, one would expect additional CNVs to be characterized in this condition, so they

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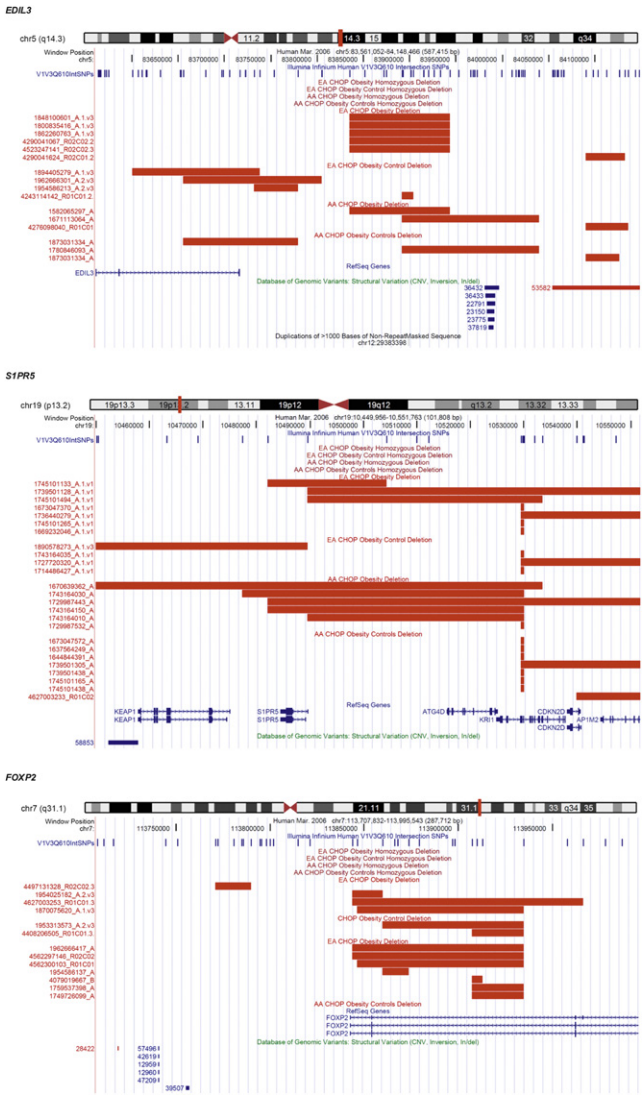

Figure 1. Visualization of Representative Loci Most Frequently Harboring Deletions
Deletions identified at the *EDIL3*, *S1PR5* and *FOXP2* loci. Vertical blue lines indicate the SNP probe coverage. Red rectangles delineate regions of copy number variation in individual cases for deletions. RefSeq gene boundaries are shown with vertical lines delineating exons. Database of Genomic Variants entries from other studies on a variety of platforms are shown as an additional control.

could collectively build up to a meaningful contribution to the missing heritably for this trait.

Taken together, the results of our unbiased approach to assessing the entire genome have revealed genes overlapping with CNVs that are exclusive to cases in two different ethnicities and, to our knowledge, have not previously been directly implicated in the context of obesity. Additional functional studies will be needed to fully characterize the function of the genes at these loci in relation to childhood obesity

Supplemental Data

Supplemental Data include seven tables and four figures and can be found with this article online at http://www.cell.com/AJHG.

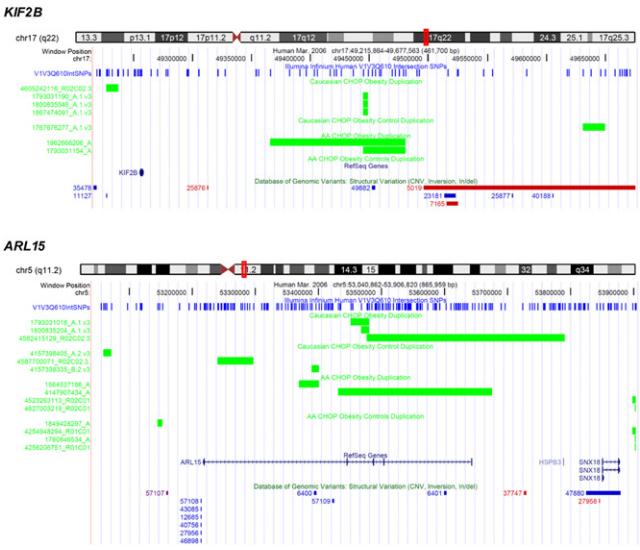

Figure 2. Visualization of Representative Loci Most Frequently Harboring Duplications
Duplications identified at the *KIF2B* and *ARL15* loci. Vertical blue lines indicate the SNP probe coverage. Green rectangles delineate regions of copy number variation in individual cases for duplications. RefSeq gene boundaries are shown with vertical lines delineating exons. Database of Genomic Variants entries from other studies on a variety of platforms are shown as an additional control.

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Web Resources

The URLs for data presented herein are as follows:

Center for Disease Control (CDC) growth charts, http://www.cdc.gov/nchs/about/major/nhanes/growthcharts/datafiles.htm
Database of Genomic Variants, http://projects.tcag.ca/variation
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway

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