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ARTICLE

A 680 kb duplication at the *FTO* locus in a kindred with obesity and a distinct body fat distribution

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Common intronic SNPs in the human fat mass and obesity associated (*FTO*) gene are strongly associated with body mass index (BMI). In mouse models, inactivation of the *Fto* gene results in a lean phenotype, whereas overexpression of *Fto* leads to increased food intake and obesity. The latter finding suggests that copy number variants at the *FTO* locus might be associated with extremes of adiposity. To address this question, we searched for rare, private or *de novo* copy number variation in a cohort of 985 obese and 869 lean subjects of European ancestry drawn from the extremes of the BMI distribution, genotyped on Affymetrix 6.0 arrays. A ~680 kb duplication, confirmed by real-time PCR and G-to-FISH analyses, was observed between ~rs11859825 and rs9932411 in a 68-year-old male with severe obesity. The duplicated region on chromosome 16 spans the entire genome-wide association studies risk locus for obesity, and further encompasses *RBL2, AKTIP, RPGRIP1L* and all but the last exon of the *FTO* gene. Affected family members exhibit a unique obesity phenotype, characterized by increased fat distribution in the shoulders and neck with a significantly increased neck circumference. This phenotype was accompanied by increased peripheral blood expression of *RBL2* with no alteration in expression of *FTO* or other genes in the region. No other duplications or deletions in this region were identified in the cohort of obese and lean individuals or in a further survey of 4778 individuals, suggesting that large rare copy number variants surrounding the *FTO* gene are not a frequent cause of obesity.
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INTRODUCTION

Recent genome-wide association studies (GWAS) have identified multiple common genetic variants that are associated with body mass index (BMI). The strongest GWAS signal for obesity is with intronic variants in the fat mass and obesity associated (*FTO*) gene, that encodes an Fe(II) and 2-oxoglutarate-dependent oxygenase.[1] The *FTO* gene is ubiquitously expressed with especially high transcript abundance in the hypothalamus and pancreas. In rodents, hypothalmic *Fto* expression increases upon food deprivation and expression is negatively correlated with that of the orexigenic galanin like peptide.[2] In mouse models, overexpression of *Fto* leads to increased food intake and obesity.[3] In contrast, $Fto^{-/-}$ mice exhibit normal energy intake but greater energy expenditure resulting in a lean phenotype.[4]

GWAS localized the effect of common variants on obesity in populations of European ancestry to intron 1 of the *FTO* gene.[5–7] Subsequent dense genotyping of this region has established that the effect of common variants is localized within a 42 kb haplotype block in intron 1.[8] Further, fine mapping studies, some of which employed sequencing in an attempt to fine map the locus, have not provided conclusive evidence beyond the signal imparted by rs9939609.[9] As for rare variants, a study by Meyre *et al*[10] sequenced all coding exons and identified a similar number of nonsynonymous variants in 1 433 lean (2.3%) and 1433 obese (2.4%) individuals, including functional mutations (R96H, R322Q). The distribution of variants hinted at an increased number in the tail of the protein but warranted further study. As such, within the *FTO* locus, only the region within intron 1 has been shown to be conclusively associated with increased risk of obesity, with the causative variant(s) remaining elusive. Carrier status of one or two copies of the risk alleles is associated with mean increases in body weight of 1.2 and 3.0 kg, respectively.[5] The *FTO* risk alleles have also been associated with increased energy intake of 125 to 280 kcal per day, a preference for energy-dense foods but no apparent effect on energy expenditure in humans.[11]

Allele specific expression analysis in five heterozygous carriers of rs9930506, tightly linked to rs9939609, demonstrated that the risk alleles associated with increased *FTO* transcript levels, suggesting that intron 1 encompasses a *cis*-regulatory site(s) regulating *FTO* expression.[12] This suggests that copy number variants at the *FTO* locus might be associated with extremes of adiposity. However, in subjects with proximal 16q duplications, including *FTO*, obesity is a common but not an inevitable feature in the background of significant psychomotor and intellectual deficits.[13]

We therefore sought to determine whether rare copy-number variants (CNVs) (<1%) at the *FTO* locus are found in subjects at the extremes of the body weight distribution, and if so, to characterize the effect of those CNVs. We identified a single large CNV spanning the *FTO* risk locus and characterized its molecular effects, as well as the clinical features of the subject and affected family members.

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MATERIALS AND METHODS

Unrelated obese subjects of European ancestry were recruited from the patient population of the University of Ottawa Weight Management Clinic. Briefly, inclusion criteria included a BMI $>35\,kg/m^2$ and a history of obesity for at least 10 years of adult life and no medical or psychiatric conditions predisposing to obesity. Unrelated lean subjects of the same ethnic background, with a lifelong BMI $\leq$ the 25th percentile for age and sex were recruited from the Ottawa community.[14] Subject characteristics for the total obese *versus* lean (OBLE) study population are shown in Table 1. Exclusion criteria included medical or psychiatric conditions and medications affecting body weight.[15]

Body weight was assessed using a Tanita electronic scale to the nearest 0.3 kg. Height was measured to the nearest 0.5 cm. BMI was defined, as weight in kilograms divided by height in meters squared (kg/m^2). Waist circumference was measured using an inelastic tape at the umbilicus level after normal exhalation to the nearest 0.5 cm, without clothes in the measurement area. Neck circumference was measured at the base of the neck, below the cricothyroid cartilage. The reading circle was held in front of the collarbone.[16]

Replication of copy number variant findings was sought in two distinct samples of 4778 subjects genotyped as part of a separate GWAS for coronary artery disease.[17] The study was approved by the Human Ethics Experimentation Committees of the Ottawa Hospital and University of Ottawa Heart Institute.

Genotyping and copy number variant analysis

DNA was extracted from white blood cells by standard methods.[18] SNP genotyping was carried out on Affymetrix 6.0 arrays with 644 846 SNPs passing strict genotyping QC (Hardy–Weinberg *P*-value (HWE) $>1\times10^{-6}$, Missing $<5\%$ and Minor Allele Frequency (MAF) $>5\%$ or Missing $<1\%$ and MAF $<5\%$). Imputation was performed using IMPUTE2 using the provided December 2010, 1000 Genomes European reference panel, based on a data freeze from August 2010, containing 566 haplotypes at 11 572 677 autosomal SNPs.[19] After imputation, $\sim$5.5 M SNPs passed post-QC measures (info >0.5, HWE $>$1e-6, Missing $<10\%$). SNP analyses were adjusted for sex and the first two principal components of ancestry as determined by EIGENSTRAT.[20] Lambdas of 1.035 and 1.027 for genotyped and genotyped plus imputed SNPs, respectively, suggested little to no test statistic inflation, where lambda is the median of the observed test statistics relative to its expectation under no association. All physical locations listed in this manuscript are for NBCI build 37/hg19 coordinates.

Copy number variation was assessed using Birdseye[21] and PennCNV[22] under default conditions. CNVs called by either program were retained if they were at least 1000 bp long, autosomal and had a copy number not equal to 2. Birdseye calls were further required to have a Lod score of at least 5 and PennCNV calls required at least 10 probes to be within the CNV call. Subjects were removed as outliers if they contained >150 CNV calls with either method.

FISH experiments

Experiments for this sample were carried out by the Cytogenomic Services at The Center for Applied Genomics, The Hospital for Sick Children, Toronto. Interphase FISH analysis was performed to confirm the presence of the duplicated region compared with a normal subject using one probe within (RP11-843H11, 16q12.2, 16:53,428,655-53,608,128, labeled in Spectrum Orange) and one probe outside (RP11-939K9, 16p13.3, 16:40,421,741-40,585,968,labeled in SpectrumGreen) the duplicated sequence. Sequential G-banding to FISH analysis was used to determine the location of the duplicated region with probes on either end of the duplication (RP11-843H11, 16q12.2, 16:53,428,655-53,608,128, labeled in SpectrumOrange; RP11-657O11, 16q12.2, chr16:53, 829,201-54,003,037, labeled in SpectrumGreen).

Table 1 Characteristics of OBLE Study Population

	Obese cases	*Lean controls*
N	993	1010
Age (years)	49.8 ± 10.7	44.7 ± 15.1
Male (%)	29.5%	39.7%
BMI (kg/m^2)	41.3 ± 8.4	20.7 ± 2.0
Waist circumference (cm)	111.7 ± 19.0	61.1 ± 10.2
Body fat (%)	46.2 ± 7.0	20.3 ± 7.0
Fat mass (kg)	54.6 ± 18.2	12.3 ± 4.4
Fat-free mass (kg)	61.9 ± 14.0	48.8 ± 10.0

Abbreviation: BMI, body mass index.
Data are provided as mean ± SD.

Fine mapping of the duplicate region

Saliva samples were obtained from available members of the extended kindred using Oragene DNA Self-Collection kit (DNA Genotek, Ottawa ON, Canada). Genomic DNA was isolated according to the manufacturer's instructions. Real-time PCR was performed using a Roche LightCyler 480 (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR Green. Primers were designed for both outside and inside of the duplication region (Supplementary Table 2). Changes in copy number were assessed using the $\Delta\Delta C_t$ method relative to a region of normal copy number. The C_t value was determined by the amount of SYBR green from quantitative PCR using LightCycler 480 (Roche). Rounds of primer design were used to fine map the breakpoints of the duplication. Long range PCR was used to determine whether or not the duplication was tandem to the original sequence.

Statistical analysis of pedigree traits

A total of 3 to 6 controls matched for age, sex and BMI were obtained for each of the affected subjects. Comparison of a trait between affecteds and controls was obtained by running linear regression of the trait on affected *versus* control status adjusted for age, sex and BMI, with the respective covariate removed if it is the trait of interest being analyzed. All statistical analyses were performed using R. (http://www.r-project.org/)

RNA expression analysis

A volume of 2.5 ml of fasting whole-blood was drawn directly into PAXgene blood RNA tubes (BD-Canada, Mississauga, ON). Isolation of total RNA was accomplished according to the manufacturer's instructions using Paxgene Blood RNA Kit (Qiagen, Toronto, ON, Canada). Primers were designed for each gene of interest (Supplementary Table 1) and expression was measured using SYBR Green on a Roche LightCycler 480. Statistical analysis of results was performed using linear regression with adjustment for age, sex and BMI. Significance was defined as a *P*-value of <0.05 after Bonferroni correction for the number of genes tested ($n=7$).

RESULTS

Single marker and copy number variant analysis

Single marker analysis confirmed previously shown associations between intronic SNPs in *FTO* and obesity in our cohort, with rs9939609 and tightly linked SNPs showing strong evidence for association comparable to previous results in similarly defined populations (rs9939609, risk allele = A (forward strand), OR (95% CI) = 1.63 (1.42, 1.87), ncase = 952, nctrl = 863, RAFcase = 47.1%, RAFctrl = 34.7, $P=6\times10^{-13}$). The Manhattan plot of this region on chromosome 16 is illustrated in Figure 1 and confirms that the risk imparted by common variants is localized to the 42 kb region in intron 1.

Following exclusion of subjects who had unnatural CNV distributions ($n=149$ individuals), we assessed the region surrounding FTO for copy number variations in 985 obese and 869 lean individuals. Analysis identified a 680 kb duplication between $\sim$53 325 073 (rs11859825) and 54 005 163 (rs9932411) in a 68-year-old male (Subject 0001) with severe obesity of childhood onset (Figure 2a). This subject was a homozygous carrier of the non-risk common (G) allele of rs9939609. The duplicated region on chromosome 16 spans the entire GWAS risk locus for obesity shown in Figure 1 and encompasses *RBL2, AKTIP, RPGRIP1L* and all but the last exon of the

FTO gene (Figure 2b). This CNV was not present in any other member of the extremes of obesity sample (OBLE) or in two distinct samples of 4778 subjects genotyped as part of a separate GWAS for coronary artery disease[17] (OHGS_A2, 1955 subjects, 2.8% BMI <20, 6.0% BMI >30, Affymetrix 500K array; OHGS_B2, 2823 subjects, 2.7% BMI <20, 6.0% BMI >35, Affymetrix 6.0 array).

Fine mapping and FISH analysis

Fine mapping and FISH analysis confirmed the original finding of the duplication and allowed us to narrow the breakpoints of the duplication from 53 322 333–53 325 358 to 54 001 784–54 005 684. Results from long range PCR revealed that the duplication was not immediately tandem or inverted.

Interphase FISH analysis also confirmed the presence of the duplication in the proband (Subject no. 0001) as compared with a normal male control, with the majority of nuclei from the proband exhibiting either an enhanced signal or two distinct signals for the probe within the duplication (RP11-834H11, SpectrumOrange) relative to another probe outside the duplication (RP11-939K9, SpectrumGreen) (Figure 3a). In sequential G-to-FISH analysis, in the majority of nuclei an enhanced signal was observed on one of the two chromosome 16 homologs, again confirming the duplication, however, no probes bound outside of 16q12.2, confirming that the duplication is in close proximity to the original copy. Owing to the limitation of the resolution, it was not possible to determine the orientation of the inserted sequences relative to each other ie, direct or inverted (Figure 3b).

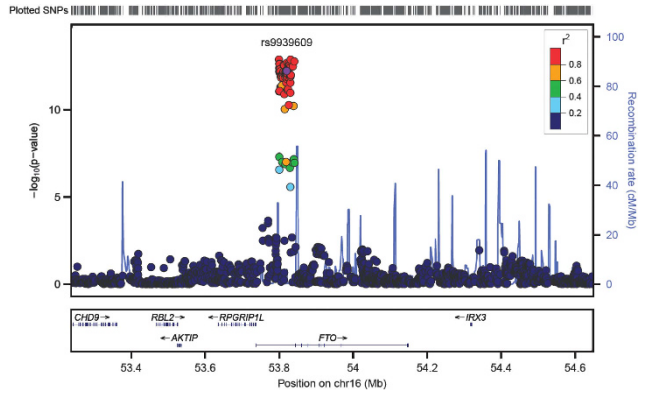

Figure 1 Manhattan plot: Obese *versus* Lean (OBLE) case control analysis. Note risk locus signal spans ~42 kb and is subsumed in duplication. rs9939609 is highlighted. This figure was generated using LocusZoom.[38]

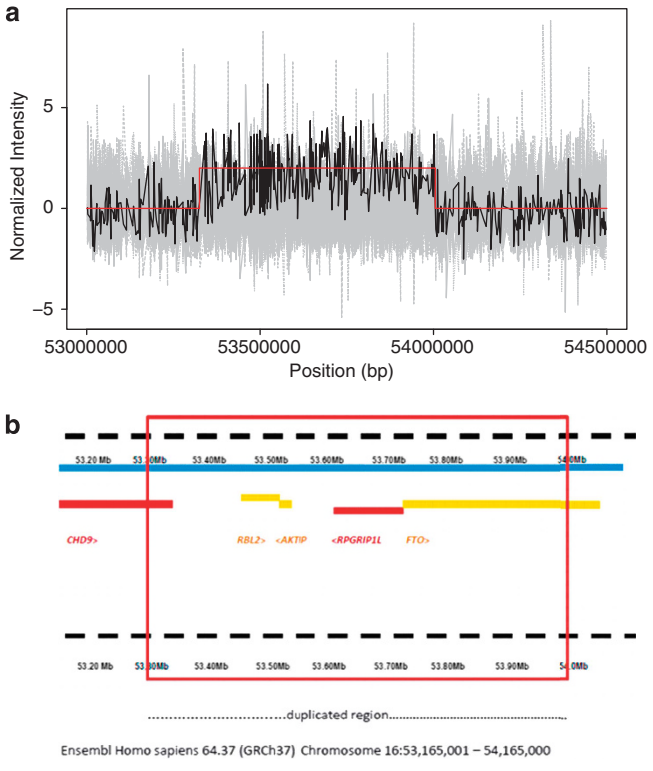

Figure 2 (**a**) Results from Birdseye for subjects on the same plate (gray) and the proband (black). In red is the result from the hidden Markov model used by Birdseye and showing the location of the 680 kb duplication. (**b**) The Duplication region (16:53, 325,073-16:54,005,163) is outlined by the red box. This region spans exon 28 of *CHD9* to intron 8 of *FTO*. It encompasses the full length of *RBL2, AKTIP* and *RPGRIP1L*.

Clinical phenotype of affected family members

Available family members of the affected subject were recruited and genotyped (Figure 4). In addition to the proband (Subject 0001), three other family members (Subjects 1001, 9000 and 9001) were

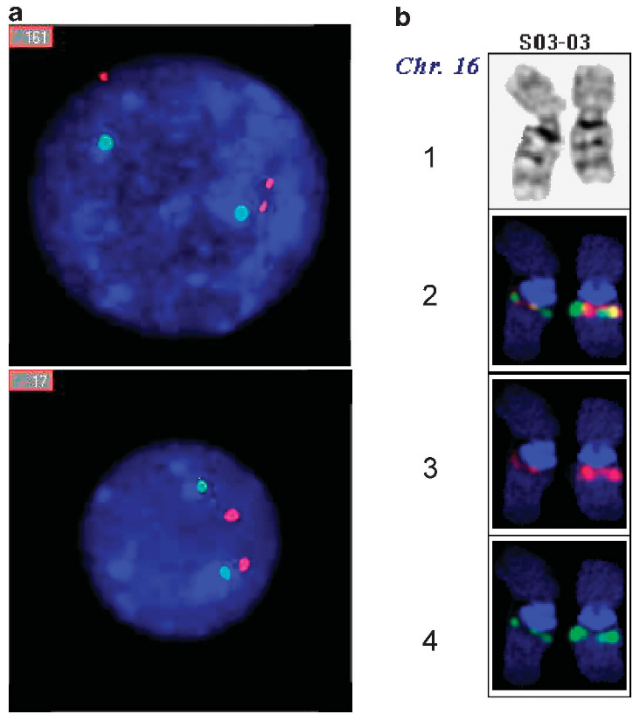

Figure 3 (**a**) Representative interphase FISH with probes for within (red) and outside (green) the duplicated region. In the proband (Subjects 0001)(top), three signals of red and two signals of green are evident *versus* two red and two green in the control (bottom), demonstrating that duplication occurs in one of two chromosomes at 16q12.2. (**b**) Representative metaphase FISH image with two probes from within the duplicated region exhibit an enhanced signal on one of the two chromosomes. First panel: G-banding of chromosome 16, second panel: the same chr16 pairs sequential to two color FISH. Third panel: Signal from RP11-834H11 alone. Fourth panel: Signal from RP11-657O11 alone. Both enhanced signals overlap on the same homolog of chromosome 16, indicating the duplicated region is located in close proximity to the original locus. Resolution is not sufficient to determine orientation (tandem or inverted).

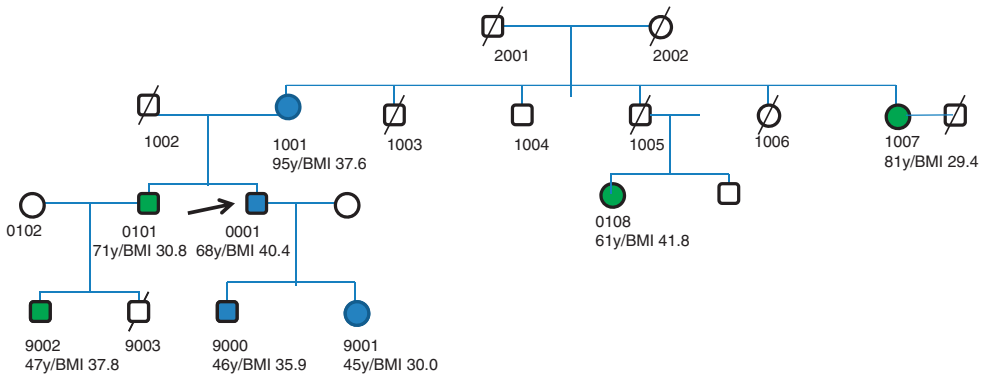

Figure 4 Affected pedigree. A total of eight subjects were available for genotyping. Four family members harboring the same CNV (Subjects 0001, 1001, 9000 and 9001) were identified (blue squares or circles). Genotyped subjects not harboring the CNV (Subjects 1007, 0101, 0108 and 9002) are denoted by green squares or circles. Age and BMI of each is indicated.

Table 2. Phenotypic comparison of obese carriers of 680 kb duplication and matched controls

Subject number	Age	Age of matched controls[a]	BMI	BMI of matched controls	Neck circumference	Neck circumference of matched controls
0001	68	67.4 ± 3.6	40.4	40.1 ± 1.0	20.5	17.2 ± 0.9
9000	46	45.8 ± 1.4	35.9	36.6 ± 1.1	20.5	17.4 ± 2.0
9001	45	45.0 ± 0.7	30.0	30.4 ± 1.1	15.0	13.9 ± 1.4
1001	95	87.5 ± 1.5	37.6	35.5 ± 2.6	14.6	14.5 ± 0.7
		$P=0.813$		$P=0.995$		$P=0.033$

Abbreviation: BMI, body mass index.
[a]Controls were matched for age, gender and BMI but not neck circumference. A total of 3–6 control subjects were matched to each affected subject

Table 3 Whole-blood mRNA expression of *FTO* and adjacent genes in carriers of 680 kb duplication *versus* matched controls

Gene	Relative mRNA expression for carriers of 680 kb duplication (mean)[a]	Relative mRNA expression for matched controls (mean)[a]	P-value*
AKTIP	3.45	2.89	9.36E-02
*CHD9*ex2-3	3.51	3.61	7.37E-01
*CHD9*ex29-30	2.42	2.14	1.37E-01
*FTO*ex3-5	2.43	2.06	1.29E-01
*FTO*ex9	2.57	2.35	5.62E-01
RBL2	3.02	1.92	2.63E-05
RPGRIP1L	5.05	3.86	4.67E-02

**P*-value from a linear regression analysis for gene expression regressed on affected *versus* control status, adjusted for age, sex and BMI. A total of 3–6 control subjects were matched to each affected subject.
[a]Relative mRNA expression is the level of mRNA expression of each gene relative to the level of mRNA expression of a house keeping gene (PPIA).

identified who each carried a single copy of the duplicated region. Three of these (no. 0001, 1001, 9000) were homozygous for the common (non-risk) allele of rs9939609 and one (no. 9001) was heterozygous for this allele. All exhibited obesity and a somewhat distinctive body fat distribution (Supplementary Figure) in the face, neck and shoulders with a significantly increased ($P=0.033$) neck circumference in comparison to 3 to 6 well matched obese non-carriers of the CNV (Table 2). Although the mean BMI of carriers ($36.0 \pm 2.2\,kg/m^2$) and non-carriers ($35.0 \pm 2.9\,kg/m^2$) of the duplication did not differ significantly, the phenotype of fat distribution in the neck and shoulders was not evident in obese or non-obese family members who were available for genotyping and who were found to be non carriers of this CNV (Subjects 1007, 0101, 0108 and 9002). Of these four non-carriers, two (no. 0101 and 0108) were homozygous for the common (non-risk) allele of rs9939609 and two (no. 9002 and 1007) were heterozygous for this allele.

In terms of disease-related characteristics, no consistent findings were noted. The proband had a diagnosis of chronic obstructive lung disease, osteoarthritis and dyslipidemia and his elderly mother, who was also a carrier of the duplication, was being treated for hypothyroidism and hypertension. The two other affected family members were in good health.

RNA expression in whole blood

RNA expression analysis from whole blood revealed no difference in expression of 5′ or 3′ *FTO* transcripts, *RPGRIP1L, AKTIP* or 3′ or 5′ *CDH9* transcripts between the carriers and the well matched non-carriers. In contrast, *RBL2* expression was greater in the small number of affected subjects ($P=3\times10^{-5}$) (Table 3).

DISCUSSION

In the mouse, over-expression of *Fto* leads to increased food intake and obesity,[3] whereas $Fto^{-/-}$ mice exhibit normal energy intake but greater energy expenditure resulting in a lean phenotype.[4] However, loss of function mutations in *FTO* are of similar frequency in obese and lean individuals, suggesting that haploinsufficiency does not protect against obesity.[10] Thus the functional relationship of *FTO* genetic variants to human obesity remains unclear. The obesity-associated *FTO* SNPs are intronic and are located adjacent to other genes, the expression or function of which could alter energy balance.[23]

DNA CNVs are an important source of genetic variability between individuals.[24] Rare CNVs have been implicated in highly penetrant forms of extreme obesity[25,26] and common CNV's generally tagged by biallelic SNPs have been associated with more moderate variation in body weight.[27–29] Proximal 16q duplications encompassing FTO have been associated with syndromic obesity with multiple developmental problems, dysmorphic features and psychomotor defects.[30]

We have identified a novel ~680 kb duplication at the *FTO* locus in a healthy obese individual who was a homozygous carrier of the non-risk common allele of rs9939609. The duplication encompasses all but the last exon of *FTO* but RNA expression analysis in whole blood in carriers and multiple matched controls revealed no difference in the expression of the *FTO* transcript. Similarly, in a recent report, a subject with an 11.45 Mb duplication on chromosome 16q, including the entire *FTO* locus, did not exhibit altered expression of *FTO*.[13] Although allele specific expression analysis of five individuals demonstrated that the obesity associated SNPs in intron 1 of *FTO* were associated with increased *FTO* expression in blood,[12] other studies revealed no effect of these SNPs on *FTO* expression in muscle or adipose tissues.[31] This suggest that the effects of common intronic SNPs in *FTO* on susceptibility to obesity may be mediated in part by altered expression of other genes in the region.

Notably, the four carriers of the 680 kb duplication described here exhibited increased peripheral blood expression of *RBL2*. It would have been optimal to obtain adipose tissue biopsies from these subjects. However, this was not possible because blood and anthropometric measurements were made in the periphery and the majority of the affected individuals lived a great distance from this medical center. Of interest, in 1 240 subjects in the San Antonio Family Heart Study, the risk allele of a lead GWAS SNP in intron 1 of *FTO*, rs8050136 ($r^2 = 1$ with rs9939609, HapMap2 CEU), was associated with increased *RBL2* expression but no difference in *FTO* expression in peripheral blood lymphocytes.[32] *RBL2/p130* is a member of the retinoblastoma (RB) family of tumor suppressor genes (encoding the retinoblastoma protein, pRB, p107 and p130) that interact with E2F to regulate transcription of many genes associated with cell cycle.[33] Relevant to obesity, *RBL2/p130* has been shown to have a role in preadipocyte proliferation and differentiation.[34] 3T3 cells derived from *RBL2/p130* deficient embryos display a very high adipocyte differentiation potential.[35] Accordingly, increased expression of RBL2 might be expected to attenuate adipocyte differentiation resulting in a phenotype of adipocyte hypertrophy in response to energy excess. However, in contrast to these findings of Jowett *et al*,[32] allelic expression analysis in blood samples from three *FTO* rs8050136 heterozygotes failed to find significant effects on *RBL2* expression.[12]

Although the number of available family members was small, carriers of the duplication exhibited obesity and a distinct pattern of fat distribution in the face, neck and shoulders. Few studies have specifically examined subcutaneous fat distribution in different regions of the body. In a large GWAS consisting of 10 557 European individuals, the FTO locus was strongly associated with subcutaneous fat ($P = 5.9 \times 10\text{E-}08$).[36] Relevant to the present report, in a study of 371 adolescents, the rs9939609 FTO SNP was associated with subcutaneous fat in the neck and frontal chest as measured by lipometry.[37]

In summary, we found a 680 kb duplication in a subject with extreme obesity and confirmed its presence in multiple family members who were similarly obese and shared an unusual body fat distribution. Results from this study suggest that *RBL2* might have a role in the observed phenotype. However, further studies are required to explore the possibility of a functional link between altered *RBL2* expression and obesity. This study also suggests that large copy number variants in this genomic region are likely to be quite rare, as only one was observed among the 2003 individuals with extreme BMIs and 4778 additional subjects investigated. Nonetheless, given that this locus imparts a major effect on obesity risk but is still poorly understood, and that large a number of subjects have been genotyped on similar arrays worldwide for a wide variety of phenotypes, it would be worthwhile to screen this locus for rare CNVs in an attempt to correlate individual copy number events with unique phenotypes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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