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The missense variation landscape of *FTO, MC4R* and *TMEM18* in obese children of African ancestry

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Abstract

Common variation at the loci harboring *FTO, MC4R* and *TMEM18* is consistently reported as being statistically the most strongly associated with obesity. We investigated if these loci also harbor rarer missense variants that confer substantially higher risk of common childhood obesity in African American (AA) children. We sequenced the exons of *FTO, MC4R* and *TMEM18* in an initial subset of our cohort i.e. 200 obese (BMI≥95th percentile) and 200 lean AA children (BMI≤5th percentile). Any missense exonic variants that were uncovered went on to be further genotyped in a further 768 obese and 768 lean (BMI≤50th percentile) children of the same ethnicity. A number of exonic variants were observed from our sequencing effort: seven in *FTO*, of which four were non-synonymous (A163T, G182A, M400V and A405V), thirteen in *MC4R*, of which six were non-synonymous (V103I, N123S, S136A, F202L, N240S and I251L) and four in *TMEM18*, of which two were non-synonymous (P2S and V113L). Follow-up genotyping of these missense variants revealed only one significant difference in allele frequency between cases and controls, namely with N240S in *MC4R*(Fisher's Exact $P = 0.0001$). In summary, moderately rare missense variants within the *FTO, MC4R* and *TMEM18* genes observed in our study did not confer risk of common childhood obesity in African Americans except for a degree of evidence for one known loss-of-function variant in *MC4R*.

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AUTHOR INFORMATION

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Keywords

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Genome wide association studies (GWAS) have revealed genomic variants strongly associated with most common disorders; indeed there is general consensus on these findings from positive replication outcomes by independent groups. The clear leader to date, with respect to strength of association, is the *FTO* locus[1]; this association with BMI and obesity has now been widely replicated by multiple independent groups. Common variants of *MC4R* have also been discovered to be strongly associated with BMI and related traits[2], complementing the already described rare coding mutations in this gene involved in monogenic obesity[3]; more than 150 missense and nonsense mutations have already been reported in *MC4R* [4-7] but have not been implicated as a frequent cause of human obesity[5, 8]. A variant located approximately 30kb downstream of *TMEM18* has also been consistently and strongly associated with BMI in GWAS reports [9].

To date, most GWAS reports have resulted from investigations of populations of European origin. Indeed, like many of the other replication efforts, *FTO* shows the strongest association with BMI in our large European American pediatric cohort[10]. However, the role of the *FTO* locus in influencing BMI and obesity predisposition in populations of African ancestry has been previously less clear, but consensus is emerging from large cohort studies, both in adults[11] and in our own pediatric cohort[12] that a common SNP can capture the association in both ethnicities. The picture is substantially less clear for *MC4R* and *TMEM18*, where further work in other ethnicities is required to fully understand their associations with BMI and obesity[13].

Investigators have hypothesized that loci revealed by GWAS may not only harbor the common variants conferring modest risk that led them there, but may also harbor rarer variants that confer substantially higher risk of the same disease. A precedent for this has already been set in this regard, where a study of ten candidate genes associated with type 1 diabetes led to the discovery of rare variants associated with the disease in the interferon induced with helicase C domain 1 (*IFIH1*) gene[14] and more recently an extensive sequencing effort of inflammatory bowel disease GWAS-implicated genes revealed such variants[15].

A French sequencing effort in Caucasians (primarily adults) has already reported a set of exonic mutations in *FTO*; however, due to the lack of significant differences in the frequencies of these variants between lean and obese individuals, this study was largely negative[16]. In addition, sequencing efforts to date on *MC4R* have been mainly limited to extreme obesity[4, 5, 7].

We reasoned that such rare disease-conferring but highly penetrant genetic variants at these loci could be easier to determine in children, where the relative environmental exposure time is substantially less. Added to that we were also in a position to investigate this issue in African American children i.e. African ancestry represents the greatest haplotype diversity so we should be able to determine the maximum number of existing exonic variants; indeed

this is the same cohort that we first established the distillation of the trans-ethnic association between obesity and *FTO*[12]. In addition we elected to investigate the next two most strongly associated loci resulting from GWAS, namely *MC4R* and *TMEM18* in a comparable fashion.

From our Sanger sequencing effort of the nine exons of the *FTO* gene at the ends of the BMI distribution of our defined cohort (200 cases and 200 lean controls) we identified a total of seven variants, three of which were synonymous (T6T, I334I and D394D) and four were non-synonymous [A163T, G182A, M400V and A405V]. G182A, D394D and M400V were not previously reported by the French study of Caucasian cases[16]. The most notable observation from this initial sequencing phase was with A405V, which was present in eleven obese (BMI≥95th percentile) cases and only four lean (BMI≤5th percentile) subjects i.e. almost three times more frequent in cases (Table 1).

A similar sequencing approach for the single exon of *MC4R* using the same cohort revealed thirteen variants (Table 1), seven of which were synonymous [G8, A135, Q156, I198 and C271, C279, L322] and six were non-synonymous [V103I, N123S, S136A, F202L, N240S and I251L]. Among the non-synonymous variants, four of them had been reported previously[5] [V103I, F202L, N240S and I251L], with the remaining two being novel [N123S and S136A].

Finally, sequencing of *TMEM18* revealed only four exonic variants, two of which were novel and synonymous [V17 and L51] and two which were non-synonymous [P2S and V113L], with the latter having already been recorded in publically available databases (rs11370572 and 1KG2669666, respectively).

We elected to follow-up all non-synonymous variants detected in these three genes to investigate the possible extent of their role in the pathogenesis of childhood obesity in African Americans in an additional 768 obese (BMI≥95th percentile) and 768 lean (BMI≤50th percentile) individuals using TaqMan genotyping; however it should be noted that we could not generate a successful genotyping assay for S136A in *MC4R*.

Analysis of the resulting genotyping data revealed that there were no significant differences in the frequency of these variants between cases and controls, including A405V in *FTO* which had looked initially promising from the sequencing outcomes, except for N240S in *MC4R* (Fisher's Exact $P = 0.0001$) (Table 2).

Our work complements recent work carried out in the French study of Caucasians[16]. We also found that missense variants in *FTO* did not play a substantial role in conferring risk for obesity in our cohort but interestingly, two of the missense variants had not been detected in that Caucasian sequencing effort i.e. G182A and M400V.

Furthermore, our sequencing effort of *MC4R* and *TMEM18* revealed variants that had not been previously published. Two novel non-synonymous variants were uncovered within *MC4R* i.e. N123S and S136A, both in the transmembrane domain. The two non-synonymous variants in *TMEM18* were P2S and V113L; P2S is located on the very N-terminus of the protein, while V113L is located in the transmembrane domain of the protein.

Again, however, these variants did not turn out to be associated with childhood obesity in African Americans, except for the N240S variant in *MC4R*.

The *MC4R* N240S missense variant is an already known loss-of-function mutation, but which has also been observed in non-obese subjects previously[17]. Although the follow-up genotyping effort indicated an exclusive presence of the rare G allele in cases only (Table 2), when combined with the discovery sequenced dataset, where there was one case and one control harboring the same allele (Table 1), the result does not strictly remain significant. As such, in order to fully resolve the role of this variant in obesity in African Americans, further studies are warranted.

So why do we not uncover more disease-conferring missense mutations in these known obesity associated loci? Apart from limited statistical power issues at the discovery stage (detection of variants only >0.5% frequency with the current strategy), it could well be that these loci only harbor a common variant that confers modest risk for common childhood obesity; on the other hand, if we had sequenced all our cases and controls, we would have been powered to detect variants down to >0.1% frequency which could confer substantial risk but we were unable to assess due to our study design. Alternatively, causative variants could be intronic or somewhat further from the initial signal than originally thought and detected via synthetic association[18]; indeed, there is still debate whether the neighboring locus to *FTO*, i.e. *RPGRIP1*, is in fact the culprit gene. Our findings should help inform future studies of these loci.

In summary, we have shown that moderately rare missense variants observed in the exons of the three genes discovered from GWAS, i.e. *FTO*, *MC4R* and *TMEM18*, do not confer risk of common childhood obesity in African Americans, except for a degree of evidence with the known N240S variant in *MC4R*. Furthermore our *FTO* findings agree with the prior studies from similar analyses in subjects of European ancestry[5, 8].

RESEARCH METHODS AND PROCEDURES

Study population

All subjects were consecutively recruited from the Greater Philadelphia area from 2006 to 2010 at the Children's Hospital of Philadelphia (CHOP). Our African American study consisted of equal numbers of obese (BMI≥95th percentile) and lean children (BMI≤5th percentile for sequencing; BMI≤50th percentile for follow up genotyping). All of these participants had their blood drawn in to a 7ml EDTA blood collection tube and were subsequently DNA extracted for genotyping. BMI percentiles were defined using the Center for Disease control (CDC) z-scores (http://www.cdc.gov/nchs/about/major/nhanes/growthcharts/datafiles.htm). All subjects were biologically unrelated and were aged between 2 and 18 years old. All subjects were between -3 and +3 standard deviations of CDC corrected BMI i.e. outliers (n<200) were excluded to avoid the consequences of potential measurement error or Mendelian causes of extreme obesity. This study was approved by the Institutional Review Board of CHOP. Parental informed consent, and child assent where appropriate, was given for each study participant for both the blood collection and subsequent genotyping.

Sequencing

PCR products corresponding to all nine exons of *FTO* were generated for 200 obese (BMI≥95th percentile) subjects and 200 lean (BMI≤5th percentile) subjects in this study. PCR Primers used are listed in Tables S1, S3 and S5. Following the PCR reactions, each product was sequenced using standard Sanger sequencing methods (Applied Biosystems Foster City, CA, USA). Analysis of the sequences and subsequent determination of exonic variants was carried out using the Sequencher 4.9 software package. Sequencing primers used are listed in Table S2, S4 and S6.

Genotyping

All missense variants observed were selected for follow up genotyping in a further 768 obese (BMI≥95th percentile) and 768 lean (BMI≤50th percentile) children. The SNPs selected were A405V, G182A, M400V and A163T in *FTO*; V103I, N123S, I251L, S136A, F202L and N240S in *MC4R* and P2S and V113L in *TMEM18*. They were genotyped using the TaqMan platform (Applied Biosystems) following standard procedures provided by the manufacturer; however we could not generate a successful genotyping assay for S136A in *MC4R*.

Analysis

We queried the data for the SNPs of interest in our pediatric sample. All statistical analyses were carried out using the Fisher's Exact Test, due to the fact that it is the most appropriate test handle association assessments of rare variants with a given trait. African ancestry was confirmed by multi-dimensional scaling in *plink*[19].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FTO	fat mass and obesity associated
TMEM18	transmembrane protein 18
MC4R	melanocortin receptor 4

GWAS genome wide association study

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Table 1

Repertoire and frequency of exonic mutations in *FTO*, *MC4R* and *TMEM18* in African Americans from the initial sequencing effort of 200 childhood obesity (BMI≥95th percentile) cases and 200 lean (BMI≤5th percentile) controls.

FTO

Exon	Type of mutation	Amino acid	# cases	# controls	Reported in Caucasians?
1	Synonymous	Thr6	22	25	yes
3	Non-synonymous	Ala163Thr	1	0	yes
3	Non-synonymous	Gly182Ala	5	3	no
6	Synonymous	Ile334	7	10	yes
7	Synonymous	Asp394	2	0	no
7	Non-synonymous	Met400Val	1	1	no
7	Non-synonymous	Ala405Val	11	4	yes

MC4R

Exon	Type of mutation	Amino acid	# cases	# controls	Reported Previously
1	Synonymous	Gly8	0	1	no
1	Non-synonymous	Val103Ile	2	3	yes
1	Non-synonymous	Asn123Ser	0	1	no
1	Synonymous	Ala135	0	1	no
1	Non-synonymous	Ser136Ala	0	1	no
1	Synonymous	Gln156	1	0	yes
1	Synonymous	Ile198	11	13	no
1	Non-synonymous	Phe202Leu	2	7	yes
1	Non-synonymous	Asn240Ser	1	1	yes
1	Non-synonymous	Ile251Leu	0	1	yes
1	Synonymous	Cys271	0	1	no
1	Synonymous	Cys279	1	0	no
1	Synonymous	Leu322	0	1	no

TMEM18

Exon	Type of mutation	Amino acid	# cases	# controls
1	Non-synonymous	Pro2Ser	2	3

TMEM18

Exon	Type of mutation	Amino acid	# cases	# controls
1	Synonymous	Val17	13	20
2	Synonymous	Leu51	0	1
5	Non-synonymous	Val113Leu	0	1

Table 2

Distribution of the four missense variants uncovered through the exonic sequencing of *FTO*, *MC4R* and *TMEM18* in African Americans through the genotyping of 768 obese (BMI≥95th percentile) and 768 lean (BMI≤50th percentile) children of the same ethnicity.

FTO

A405V (*P* = 0.4472)			G182A (*P* = 0.5693)			M400V (*P* = 1.0000)			A163T (*P* = 0.6455)		
Alleles	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**
CC	739	732	GG	737	742	AA	756	758	GG	755	757
CT	27	35	CG	27	23	AG	3	3	AG	10	9
TT	1	0	CC	0	0	GG	0	0	AA	0	0

MC4R

F202L (*P* = 0.5270)			N240S (*P* = 0.0001)			N123S (*P* = 1.0000)			I251L (*P* = 1.0000)			V103I (*P* = 0.2411)		
Alleles	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**
CC	711	712	AA	568	664	AA	743	689	AA	730	703	CC	662	685
CA	18	23	AG	3	0	AG	0	0	AC	4	3	CT	13	22
AA	0	0	GG	0	0	GG	0	0	CC	0	0	TT	1	0

TMEM18

P2S (*P* = 0.3983)			V113L (*P* = 1.0000)		
Alleles	**# cases**	**#cntrls**	**Alleles**	**# cases**	**#cntrls**
GG	753	738	GG	747	747
GA	9	13	GA	0	1
AA	0	0	AA	0	0