Association study between near-MC4R variants and obesity-related variables in Portuguese young adults

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ABSTRACT

The aim of this study was to investigate in a population sample of Portuguese young adults i) the association of near-MC4R variantswith obesity measures; and ii) the presence ofmutations inMC4R coding region contributing to severe obesity. Polymorphisms rs17782313, rs12970134 and rs9944545 were genotyped in 544 subjects (225 males, 319 females; mean age 20.7 years) by TaqMan assay. Body Mass Index (BMI) was calculated (kg/m2) and percentage of body fat (%FAT) was measured using bioimpedance analysis. The coding region ofMC4R gene was examined in nine severe obese subjects (BMI N 35 kg/m2) by Sanger sequencing. Linear regression showed no associations between variants rs17782313, rs12970134 and rs9944545 and BMI (P = 0.459, P = 0.691 and P = 0.611, respectively) or %FAT (P= 0.853, P = 0.678 and P = 0.434, respectively). The logistic regression, in the additive model, revealed no statistically significant interactions with overweight/obesity for any SNP (P = 0.717, P=0.771 and P=0.417, respectively). Also, haplotype analysis revealed no significant associations testing for BMI, %FAT or the obesity status. Genomic DNA sequencing of the MC4R coding region revealed no variations explaining the severely obese phenotype. As main conclusion, this study in young Portuguese adults does not replicate previous findings evidencing the association of near-MC4R polymorphisms with human obesity. Nevertheless, the obtained data is in accordance with several studies reporting no such associations.

Key-words: MC4R polymorphisms; rs17782313; rs12970134; rs9944545; Obesity; Physical activity; Portugal

1. Introduction

Multiple genetic loci were robustly associated with obesity and obesity-related measures in the last decade by large-scale genome wide association studies (GWAS), most in populations of European ancestry (Lu and Loos, 2013; Albuquerque et al., 2015). Several independent GWAS, across populations worldwide have shown obesogenic effects of single nucleotide polymorphisms (SNPs) located downstream of the melanocortin-4 receptor (MC4R) gene, although other studies revealed no such associations (Loos et al., 2008; Thorleifsson et al., 2009; Xi et al., 2012; Muller et al., 2014; Srivastava et al., 2016; Bradnová et al., 2015; Logan et al., 2015). The MC4R obesity-associated variants lie about 100 to 200 kb downstream of the single coding sequence. Among these variants the rs17782313 (Loos et al., 2008) and rs12970134 (Logan et al., 2015) were studied most often. The SNP location and patterns of phenotypic associations are consistent with effects mediated through disruption of the transcriptional control of MC4R.

Moreover, mutations in MC4R are the leading cause of monogenic obesity in humans (Hinney et al., 2013). The MC4R is a 332-amino acid protein encoded by a single exon on chromosome 18q22 (Gantz et al., 1993). The gene is widely expressed in the human central nervous system including the hypothalamus and has a primary function of egulating food intake following the binding of the alpha melanocyte stimulating hormone (α-MSH) agonist enabling the production of a satiety signal (Tao, 2010). Heterozygous mutations in MC4R are present in 2– 6% of human severe obesity cases and represent the most frequent genetic cause of early-onset and morbid obesity (Hinney et al., 1999; Farooqi et al., 2003). Until now, N150 variants were described, most leading to haplo-insufficiency of the receptor consistent with the dominantly inherited pattern (Hinney et al., 2013; Wang and Tao, 2011).

Two common MC4R variants p.Val103Ile (heterozygous frequency 2–9%) and p.Ile251Leu (heterozygous frequency 0.41–1.21%) lead to an increased gene function having a slight weight-loss effect (Hinney et al., 2013; Stutzmann et al., 2007).

The aim of this study was to investigate in a population sample of Portuguese young adults i) the association of previously described near-MC4R variants with obesity and adiposity markers (BMI) and body-fat percentage (%FAT); ii) the presence of mutations in MC4R coding region contributing to severe obesity.

2. Methods

2.1. Anthropometry

The present sample included 544 healthy young adults of European descent, mainly from the central region of Portugal, being 225 males (41.4%) and 319 females (58.6%) aged between 17 and 36 years (mean age 20.68 years). Participants were randomly recruited from students at the University of Coimbra between 2013 and 2015.

Anthropometric measures weight and height were taken using a SECA scale and stadiometer, and BMI was calculated (kg/m2). Cut-off points defined by WHO were used to define underweight (BMI b 18.5 kg/m2), normal weight (18.5 ≤ BMI b 25 kg/m2), overweight (25 kg/m2 ≤ BMI b 30 kg/m2) and obesity (BMI ≥ 30 kg/m2). Body fat composition was measured using the bioelectric impedance method (BODYSTAT 1500MDD) and cut-off points to define high percentage of fat were defined based on ranges for “normal” population groups used in the software program as follows: age 10–19 years: males 18%, females 25%; age 20–30 years: males 18%, females 26% (Bodystat 1500MDD: User Guide for Body Composition Analysis). BMI data were available for 544 individuals and body fat composition for 513.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Informed consent was obtained from all patients for being included in the study.

2.2. Genotyping

Three near-MC4R SNPs were chosen for this study: the two most often analysed rs17782313 (mapped 188 kb downstream of MC4R) and rs12970134 (mapped 154 kb downstream of MC4R), and a third signal rs9944545 (mapped 80 kb downstream MC4R) recently identified (Locke et al., 2015).

A buccal swab sample was collected from each subject and genomic DNA was extracted by using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen® Biotech Corp, Tawain). SNPs rs17782313, rs12970134 and rs9944545 were genotyped by TaqMan assay (Applied Biosystems™ TaqMan™ Assays).

Additionally, nine the severely obese (BMI N 35 kg/m2) subjects (4 females and 5males) were screened for mutations in the MC4R gene, including the promoter region. Genomic DNA was amplified by polymerase chain reaction (PCR), using primers and conditions previously published for the MC4R coding sequence (Vaisse et al., 2000) and primers 5′-CGCCTACAGCCCCTAACACT-3′ (forward) and 5-CCTCCTGGGTCAGGGAGT-3′ (reverse) for the promoter region. PCR amplified fragments were purified with ExoSAP-IT (GE Healthcare, New Jersey, USA) and both forward and reverse strands were subsequently subjected to Sanger's dideoxy chain termination sequencing reaction using the Big-Dye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and processed using an ABI 3130 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing data were compared with the MC4R gene normal sequence (ENSG00000166603). The p.Val103Ile (rs2229616) common variant was screened in 100 individuals by RFLP according Gotoda et al. (1997) using modified primers and the restriction endonuclease HINCII.

2.3. Computational (in silico) predictive programs

We evaluated the probable phenotypic effect of amino acid changes using in silico tools commonly used for missense variant interpretation, including: PolyPhen2 (Adzhubei et al., 2010) (http://genetics.bwh.harvard.edu/pph2/), Mutation Assessor (Reva et al., 2007) (http://mutationassessor.org), PROVEAN (Choi and Chan, 2015) (http://provean.jcvi.org/index.php), SIFT (Kumar et al., 2009) (http://sift.jcvi.org) and MutPred v.1.2 (Li et al., 2009) (http://mutpred.mutdb.org), using the coordinates ENST00000299766 and UniProtKB - P32245.

2.4. Statistical analysis

Allelic and genotypic frequencies were estimated by direct counting. Hardy-Weinberg equilibrium P-value was achieved using an exact test. We applied linear regression models to test the association of individual SNPs with obesity-related quantitative traits BMI and fat percentage. The association between genotype distributions and overweight/obesity was tested by logistic regression under an additive genetic model, unadjusted and adjusted for sex and age, and presented as odds ratios (OR) with 95% confidence intervals (CI) and P-values. These statistical analyses were done using the set-based tests implemented on PLINK software v.1.07 (Purcell et al., 2007) (http://pngu.mgh.harvard.edu/purcell/plink/). P-values b 0.05were considered as statistical significant. QUANTO, v.1.2.4 power calculator (http://biostats.usc.edu/Quanto.html/) was used to estimate the power of association assuming an additive model (Gauderman, 2002) [27].

3. Results

3.1. Population characteristics

Table 1 summarises population characteristics of the studied sample. Among the total participants (N=544), 58.6% were females and 41.4% were males. The sample included 23 underweight subjects (4.2%), 399 normal-weight (73.3%), 94 overweight (17.3%) and 28 obese subjects (5.1%).

3.2. Population genetics

The total genotyping success rate was 0.974 for the three studied polymorphisms rs17782313, rs12970134 and rs9944545. Genotypes and allele frequencies are detailed in Table 2. Minor allele frequencies were rs17782313 C:0.184, rs12970134 A:0.199, rs9944545 T:0.247. The genotype distributions were in Hardy-Weinberg equilibrium for the three polymorphisms (P = 0.311, P = 0.501 and P = 0.196, respectively).

SNPs rs17782313 and rs12970134, distant 33.7 kb, are in high linkage disequilibrium (LD), R2 = 0.68 in our study population. SNP rs9944545 is in low LD with rs12970134 (distant 73.5 kb) (R2=0.14) aswell aswith rs17782313 (distant 107.2 kb) (R2=0.08). Eight haplotype combinations were statistically derived from the three SNPs, the most frequent were TGC (0.663), TGT (0.118) and CAT (0.101). The less frequent haplotype was CGT (0.0019).

3.3. Associations with measures of obesity

BMI and fat percentage (%FAT) quantitative traits were tested for associations with each of the studied polymorphisms, in a linear regression framework, and no significant effects were observed between variants rs17782313, rs12970134 and rs9944545 and BMI (P= 0.459, P = 0.691 and P = 0.611, respectively) or %FAT (P= 0.853, P = 0.678 and P=0.434, respectively) (Table 2).Moreover, no significant association was obtained for derived haplotypes combining the three polymorphisms (data not shown).

Association betweenMC4R SNPs and the obesity risk was also tested under a case-control design, merging overweight and obese subjects in one group. The logistic regression, in the additive model, revealed no statistically significant associations with overweight/obesity for the individual MC4R polymorphisms (P=0.717, P=0.771 and P=0.417, respectively) (Table 3). Also, the haplotype analysis combining the three polymorphisms, revealed no significant association testing neither for all haplotype effects ($\chi 2$ = 0.014; P = 0.90) nor for individual haplotypes (Table 3). In the same way, establishing a case group with high percentage ranges of fat, no significant associations were obtained for individual SNPs or derived haplotypes (data not shown).

3.4. Interaction between MC4R polymorphisms and physical activity

As in this same cohort of individuals we showed that physical activity attenuates the genetic susceptibility to obesity for polymorphisms in FTO gene (Muc et al., 2015), we tested for GxE effects but no significant interaction between individual polymorphisms and physical activity that affects neither BMI nor fat percentage was detected, although a near significant interaction affecting %FAT was observed for rs12970134 (Pinteraction=0.08).

3.5. MC4R mutations screening

Genomic DNA sequencing of theMC4R coding region and adjacent promoter regions of nine severe obese subjects, revealed no variations except the previous described common transversion c.751ANC (p.Ile251Leu) (rs52820871) in a male with a BMI of

49.01 kg/m2. A bioinformatic analysis using in silico tools to predict whether this missense change is damaging to the resultant protein function showed the following outputs (score/prediction): PolyPhen-2: 0.001/benign; MutationAssessor:−2.02/neutral; PROVEAN: 1.778/neutral; SIFT 1.000/Tolerated; MutPred: probability of being deleterious 0.332. The common p.Val103Ile polymorphism(rs2229616)was not found in any of the nine severe obese subjects. Moreover, the screening of 100 random individuals enabled to found this variant in only one subject allowing a 0.01 frequency of heterozygous subjects. For this missense change the bioinformatic analysis to predict whether this missense change is damaging showed (score/prediction): PolyPhen-2: 0.025/benign; MutationAssessor: 1.065/low; PROVEAN: −0.07/neutral; SIFT 0.253/Tolerated; MutPred: probability of being deleterious 0.192.

4. Discussion

The melanocortin 4 receptor (MC4R) gene is known to be the most common cause of monogenic obesity, but its flanking genomic region has also been implicated in common obesity. The most often studied near-MC4R polymorphisms, rs12970134 and rs17782313, were found to be associated with obesity and obesity-related traits in several studies in Asian and European populations, both in children and adults. Also in a sample of Portuguese children significant associations were found between MC4R rs12970134 and BMI, BMI Z-score and WC, as also with the risk of obesity, but the direction of effect for the risk allele was reverse when comparing with that in the original reports (Albuquerque et al., 2014).

However the genetic association results have been often inconsistent (see the Meta-Analysis of Xi et al., 2012), and replication studies are still necessary across different populations, age groups or genders. In this work we evaluated the association between three near-MC4R polymorphisms, rs17782313, rs12970134 and rs9944545, and overweight-obesity and measures of obesity in Portuguese young adults. No statistically significant associations were found between individual polymorphisms or derived haplotypes with risk of overweight/obesity or obesity quantitative traits BMI and percentage of fat. This absence of significant associations in comparison with other studies in European populations could be the result of a limited statistical power (that is the chance of detecting true effects), consequence of a lower sample size or a modest effect size of polymorphisms.

The estimated power of association in the study population, assuming the simplest genetic model of a single diallelic disease mutation, was about 10% for rs17782313 and rs12970134 polymorphisms and about 20% for rs9944545, far away from the power value of 80% commonly considered as sufficient to avoid false negative associations. Thus, a

larger sample size could be required to achieve sufficient statistical power improving the ability of disease prediction for MC4R polymorphisms in the Portuguese population. Differences in genetic backgrounds of studied populations are also strong candidates to explain heterogeneity in disease-association studies (Myles et al., 2008). As an analysed SNP could be in LD with a true causal variant, if replication of the index SNP is tested in a second population with a different genetic background, it is possible that a change in LDwill result in a weaker or absence of association between the index SNP and the obesity trait (Lu and Loos, 2013). Differences in allele frequencies can be observed for MC4R SNPs rs17782313 and rs12970134 between the Portuguese population (MAF 0.184 and 0.199, respectively) and populations from GBR (British) (MAF 0.236 and 0.239, respectively), IBS (Iberian Peninsula in Spain) (MAF 0.257 and 0.289, respectively) or TSI (Toscani in Italy) (MAF 0.280 and 0.299, respectively) (data

reported in 1000G, Ensembl), ranging statistical significant differences between Portugal and some of these populations. Thus, we hypothesize that allele frequency heterogeneity, may explain the absence of significant association results in the Portuguese population in comparison with other populations.
Genomic DNA sequencing of the MC4R coding region of severe obese subjects, revealed no variations explaining the obese phenotype. The p.Ile251Leu variant was found in an individual with BMI of 49.01 kg/m2. However, this variant, with a frequency b 0.01 in European populations (data from1000G, Ensembl), is unlikely to explain the severe obesity in this individual as it was reported functionally silent and present in both normal weight and obese populations (Hinney et al., 2013). In concordance, the bioinformatic analysis using several silico tools predict that the missense change p.Ile251Leu is neutral. Furthermore, a meta analysis by Stutzmann et al. (2007) showed a protective effect of the 251Leu allele (reduced by nearly 50% risk of obesity for carriers of this allele when compared with wild-type allele carriers).
In conclusion, this study in young Portuguese adults do not replicate previous findings evidencing the association of near-MC4R polymorphisms rs17782313 C/T, rs12970134with human obesity. Nevertheless, the obtained data is in accordance with several studies reporting no such associations.

The authors declare that they have no conflict of interest.

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Table 1 Characteristics of the study sample and comparison between the sexes.

Variables	Total	Males	Females
N (%)	544	225 (41.4)	319 (58.6)
Age (years)	20.68 (2.65)	21.16 (3.17)	20.50 (2.46)
Weight (kg)	64.32 (12.64)	72.04 (12.23)	58.44 (9.37)
Height (m)	1.67 (0.09)	1.74 (0.07)	1.61 (0.06)
BMI (kg/m2)	23.17 (4.85)	23.79 (4.05	22.72 (3.62)
Body mass (%)	22.81 (7.50)	16.36 (4.64)	27.73 (5.18)
Underweight (BMI < 18.5 kg/m2)*	23 (4.2)	5 (2.2)	18 (5.6)
Normal weight (18.5 ≤ BMI <25 kg/m2)*	399 (73.3)	159 (70.7)	240 (75.2)
Overweight (25 ≤ BMI < 30 kg/m2) *	94 (17.3)	49 (21.8)	45 (14.1)
Obesity (BMI ≥30 kg/m2) *	28 (5.1)	12 (5.3)	16 (5.0)

Abbreviations: BMI, body mass index; N, number of individuals. Data presented as mean (standard deviation) for continuous anthropometric variables and as N (%) for categorical variables (*).

Table 2 Genotype and allele frequencies for the three MC4R polymorphisms in the sample of Portuguese young adults and their and their associations with obesity-related quantitative traits BMI and percentage of body fat.

SNP	Genotypes	Alleles	HWE P	Trait	N	β (SE)	P
rs17782313 Chr18:57,851,097	CC:14 (0.027) CT:166 (0.315) TT:346 (0.658)	C:0.184 T:0.816	0.311	BMI %FAT	526 495	0.234(0.316) - 0.117(0.633)	0.459 0.853
rs12970134 Chr18:57,884,750	AA:24 (0.044) AG:168 (0.310) GG:350 (0.646)	A:0.199 G:0.801	0.501	BMI %FAT	542 511	0.115(0.289) - 0.241(0.580)	0.691 0.678
rs9944545 Chr18:57,958,244	TT:26 (0.050) TC:206 (0.395) CC:289 (0.555)	T:0.247 C:0.753	0.196	BMI %FAT	521 490	0.147(0.288) 0.449(0.575)	0.611 0.434

Abbreviations: BMI, body mass index; %FAT, body fat percentage; N, number of analysed samples.
Table includes the effect sizes (regression coefficient beta, β) of the minor allele, obtained by linear regression under an additive model, standard error (SE) and P-values (asymptotic P-value unadjusted) for quantitative traits.

Table 3 Association of the MC4R polymorphisms and derived haplotypes with the risk of overweight-obesity in the sample of Portuguese young adults (122 cases and 422 controls; 225 males, 319 females).

SNP	N	MAF (Normal group)	MAF (OB/OW group)	OR (95% CI)	P
rs17782313	526	C: 0.182	C: 0.191	1.073 (0.733-1.569)	0.717
rs12970134	542	A: 0.201	A: 0.193	0.948 (0.665-1.353)	0.771
rs9944545	521	T: 0.242	T: 0.267	1.153 (0.817-1.626)	0.417
Haplotypes		Normal group	OB/OW group	CHISQ	P
All effects		-	-	5.961 (DF 6)	0.427
CAT		0.091	0.102	0.284 (DF 1)	0.593
TAT		0.027	0.011	2.124 (DF 1)	0.145
TGT		0.121	0.150	1.374 (DF 1)	0.241
CAC		0.076	0.061	0.658 (DF 1)	0.417
TAC		0.011	0.011	0.001 (DF 1)	0.975
CGC		0.014	0.026	1.731 (DF 1)	0.188
TGC		0.659	0.639	0.342 (DF 1)	0.558

Abbreviations: MAF, Minor allele Frequency; OB/OW, Obese/Overweight; OR, odds ratio; CI, confidence interval; N, number of samples / alleles; F, females; M, males; na, not applicable.
For individual SNPs, the OR and P-values were obtained by logistic regression under an additive model considering the minor allele as the reference.
For haplotype frequencies/phases (MHF $\geq$ 0.01). Haplotype CGT was found with a frequency of 0.0019.