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WNT10B mutations in human obesity

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Abstract

Aims/hypothesis—Recent studies suggest that wingless-type MMTV integration site family, member 10B (WNT10B) may play a role in the negative regulation of adipocyte differentiation in vitro and in vivo. In order to determine whether mutations in *WNT10B* contribute to human obesity, we screened two independent populations of obese subjects for mutations in this gene.

Subjects and methods—We studied 96 subjects with severe obesity of early onset (less than 10 years of age) from the UK Genetics of Obesity Study and 115 obese Italian subjects of European origin.

Results—One proband with early-onset obesity was found to be heterozygous for a C256Y mutation, which abrogated the ability of WNT10B to activate canonical WNT signalling and block adipogenesis and was not found in 600 control alleles. All relatives of the proband who carried this allele were either overweight or obese. Three other rare missense variants were found in obese probands, but these did not clearly cosegregate with obesity in family studies and one (P301S), which was found in three unrelated subjects with early-onset obesity, had normal functional properties.

Conclusions/interpretation—These mutations represent the first naturally occurring missense variants of *WNT10B*. While the pedigree analysis in the case of C256Y WNT10B does not provide definitive proof of a causal link of this variant with obesity, the finding of a non-functioning *WNT10B* allele in a human family affected by obesity should encourage further study of this gene in other obese populations.

Keywords

Adipocyte; Adipogenesis; Human; Obesity; WNT

Introduction

Differentiation of preadipocytes into adipocytes is regulated by a balance of local and endocrine factors that either stimulate or inhibit differentiation [1]. Wingless-type MMTV integration site family members (WNTs) are a family of autocrine and paracrine growth factors that signal through multiple pathways to control numerous cellular processes, including differentiation [2]. In the best-characterised canonical pathway, binding of WNTs to frizzled receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) co-receptors leads to stabilisation of cytosolic β-catenin. The latter subsequently translocates to the nucleus, where it binds to the lymphoid enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF) family of transcription factors to activate WNT target genes.

Very little is known about the structure of WNTs as they are particularly hydrophobic because of palmitoylation of a conserved cysteine, and until recently they had never been isolated in active form. Enzymatic removal of the palmitate or site-directed and natural mutations of the modified cysteine result in loss of activity, indicating that the lipid is important for signalling [3]. Nonetheless, all WNTs share a highly conserved distribution of 23–24 cysteine residues, suggesting that WNT protein folding may depend on the formation of multiple intramolecular disulphide bonds [4].

WNTs have recently been implicated in the regulation of murine adipogenesis in vitro and in vivo. It has been shown that WNT signalling maintains preadipocytes in an undifferentiated state by inhibition of CCAAT/enhancer binding protein α (CEBPA) and peroxisome proliferator activated receptor γ (PPARG) [5, 6]. Disruption of WNT signalling results in spontaneous adipogenesis. The best candidate for the endogenous inhibitory WNT is *Wnt10b* since it is highly expressed in preadipocytes and stromovascular cells and rapidly suppressed following induction of differentiation. Transgenic mice expressing *Wnt10b* under the control of the fat-specific *Fabp4* promoter display a 50% reduction in total body fat without lipodystrophic diabetes and resist high-fat diet-induced obesity [7].

As human obesity and/or fat distribution may be influenced not only by factors controlling energy balance but also by factors affecting energy partitioning into fat versus non-fat tissues, it is plausible that inherited variants in the adipose WNT signalling system might be aetiologically linked to some subtypes of human obesity. To date, naturally occurring mutations in the gene encoding WNT10B in humans have not been described. In this study we screened the *WNT10B* gene for mutations in two independent populations of obese human subjects and, having detected novel variants, examined these for cosegregation with obesity in available family members. Additionally, we analysed the functional properties of a subset of these variants.

Subjects and methods

Subjects

Two independent populations of obese subjects were studied. Written informed consent was obtained from all subjects before enrolment and the appropriate research ethics committees approved the studies.

Group A—In this group, 96 subjects with severe obesity of early onset (<10 years of age) from the UK Genetics of Obesity Study (GOOS) were studied [8]. To increase the number of subjects whose obesity may have been due to mechanisms other than increased food intake, a group of probands without a history of hyperphagia were chosen for this study. Eighty-eight per cent were of European origin and 52% were male. BMI standard deviation scores were calculated using the UK 1990 growth reference data [9]; the mean BMI standard deviation score of the 96 patients studied was 4.3±0.9. Any variants identified in this group were examined in 100 control subjects from an ethnically matched population [10].

Group B—A total of 115 unrelated obese Italian subjects of European origin (33 men and 82 women, mean age 33.5±10.9 years, mean BMI 36.4±5.6 kg/m^2) were studied. *WNT10B* variants detected in this cohort were examined in 200 non-obese, non-diabetic subjects of the same nationality and origin (64 men, 146 women, mean age 34.02±13.2 years, mean BMI 23.2±3.2 kg/m^2).

Mutation analysis

Mutations in exons and intron–exon boundaries were sought using denaturing HPLC (DHPLC) of PCR products (Transgenomic, Omaha, NE, USA). The coding region of the *WNT10B* gene (accession no. NT_009526) was amplified by means of PCR using the following primers: forward-1 5′-CTGAACCCGCATCAAGTCTC-3′, reverse-1 5′-CGATGTGCAGACCCTGAAG-3′; forward-2 5′-CC AACACCGTGTGCTTGAC-3′, reverse-2 5′-CGGGGAA TTCCAGGAGAG-3′; forward-3 5′-TCAGCTGCCTGTC AACCTTA-3′, reverse-3 5′-TCAAACTCTAACCAGGCC TCA-3′; forward-4 5′-GTGCCTCTGTGTTCTGTCCA-3′, reverse-4 5′-CAGAGCAAAGGGCTGAAAAG-3′. Abnormal DHPLC conformers were subjected to direct sequencing using the ABI-Prism kit (Applied Biosystems, Warrington, UK).

Plasmids and mutagenesis

Full-length cDNA encoding human WNT10B was generated from HeLa cell RNA by RT-PCR using the following primers: forward, 5′-CACTGGAGGTCCTGATCGATC-3′, reverse, 5′-CAGCCCCAAGGTAAGGCTGAC-3′ as previously described [11]. PCR products were cloned into pGEM-T-easy (Promega, Madison, WI, USA), and integrity was confirmed by sequencing. WNT10B C256Y and WNT10B P301S were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and subsequently subcloned into pBabe-Puro.

Culture, differentiation, and infection of 3T3-L1 preadipocytes

3T3-L1 cells were cultured and differentiated into adipocytes as described previously [12]. 3T3-L1 cell lines producing human wild-type, C256Y and P301S WNT10B and an empty vector (EV) control 3T3-L1 cell line were generated using the pBabe-Puro retroviral vector system (Addgene, Cambridge, MA, USA) as described previously [13]. Cells were kept in puromycin-containing medium except during differentiation experiments.

TaqMan quantitative real-time reverse transcription PCR

RNA preparation, reverse transcription and conditions for TaqMan real-time RT PCR were performed as described previously [14]. Primers and probes were designed using Primer Express software (Applied Biosystems) and sequences from the GenBank database and are available upon request. Primers and probe for 18S internal control were purchased from Applied Biosystems.

Western blot analysis

Cells were washed with cold PBS and scraped into hypotonic lysis buffer as described [15] to obtain cytosolic protein extracts. After centrifugation at 4°C at 10,000 *g* for 10 min, equal amounts of protein were dissolved in Laemmli buffer, heated to 95°C and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and immunoblotted with anti-β-catenin antibody (Transduction Laboratories, Oxford, UK). Anti-mouse secondary antibody was purchased from Dako (Glostrup, Denmark).

WNT-responsive promoter reporter assay

3T3-L1 cells producing wild-type, C256Y and P301S WNT10B and EV control 3T3-L1 cells were seeded in 24-well plates (2.5×10^4 cells per well). The following day cells were transfected with 1 μg/well of the TOP-Flash promoter-reporter gene construct using FuGENE (Roche Applied Science, Indianapolis, IN, USA). To correct for transfection efficiency, 10 ng/well pRL-CMV (Promega) was cotransfected. Forty-eight hours after transfection, cells were lysed in passive lysis buffer (Promega) and luciferase activity was determined by luminometry (EG Berthold, Bad Wildbad, Germany) using the dual luciferase reporter assay (Promega).

Statistical analysis

All results are presented as mean±SD. Statistical significance was assessed using the Mann–Whitney test.

Results

Identification of WNT10B variants

We identified a number of synonymous and non-synonymous variants in the two obese populations (Table 1). In group A (early-onset obesity) two non-synonymous variants were detected. C256Y was found in heterozygous form in one UK proband of European origin and P301S was found in heterozygous form in three unrelated probands of European origin. Neither variant was identified in 100 control subjects from an ethnically matched population. Cysteine 256 is absolutely conserved among all WNT family members from *Caenorhabditis elegans* to humans (Fig. 1a). In family studies, both the father and sister of the proband were also carriers of the variant and both were overweight (28 kg/m^2) (Fig. 1b). The sister of the proband also had nearly double the percentage of body fat (measured by dual-energy X-ray absorptiometry) predicted from age-, sex- and weight-specific equations (41 versus 24%, respectively,). The proband did not display hyperphagia in the free-living

setting or show increased food intake when tested with an ad libitum test meal of 18 MJ [16]. She consumed 38 kJ/kg lean body mass; control subjects, by comparison, consumed 50 kJ/kg lean body mass. Basal metabolic rate, measured by indirect calorimetery, was comparable to that predicted using age- and sex-specific equations (data not shown). Fasting insulin was 138 pmol/l (47.1 and 60.4 pmol/l in the father and sister of the proband, respectively,). The P301S mutation involves a non-conserved amino acid residue and this variant did not segregate with early-onset obesity in the three families studied (Fig. 1b). Nonetheless, it is notable that the subject carrying the variant in homozygosity exhibited the highest BMI and, with the exception of one individual, all probands harbouring this mutation were either overweight or obese.

In group B we found one silent variant, H353H, and two heterozygous non-synonymous variants, I285T and H77Y, each in only one obese subject. Regarding the non-synonymous variants, neither amino acid residue is highly conserved. In the case of I285T only one sister was available for study; she was not obese and was wild-type at this locus (Fig. 1c). In the case of H77Y the affected proband inherited the mutation from her father, who was homozygous for the variant and overweight, but her sister, who was heterozygous for the mutation, was of normal weight (Fig. 1c). Neither variant was found in 200 matched Italian controls.

Effects of C256Y and P301S mutations on the signalling ability of WNT10B

Of the variants found, we chose to study the functional properties of C256Y and P301S. The former was chosen because of the strong structure-based prediction that this would disrupt function; the latter was chosen because it was found in three unrelated probands and in no control subjects of European origin. To examine the functional consequences of the WNT10B C256Y and P301S mutations, we generated 3T3-L1 cells to constitutively produce human wild-type, C256Y and P301S WNT10B. Firstly, we determined the ability of the mutants to activate canonical WNT signalling. Both control (empty vector) and *WNT10B*-expressing preadipocytes were induced to differentiate and cytosolic protein extracts were collected at the times indicated (Fig. 2a). As expected, overexpression of wild-type *WNT10B* led to a robust increase in cytosolic β-catenin throughout the differentiation time course. Similarly, constitutive production of WNT10B P301S also resulted in stabilisation of cytosolic β-catenin. In contrast, WNT10B C256Y failed to activate canonical WNT signalling despite gene expression being at a similar level to wild-type and P301S WNT10B (Fig. 2g). This was further confirmed with promoter assays using the luciferase reporter construct TOP-Flash (Fig. 2b). This reporter contains multiple TCF binding sites and represents a readout for β-catenin transcriptional activity. Constitutive production of wild-type and P301S WNT10B led to two-fold activation of TCF promoter activity. In contrast, WNT10B C256Y failed to induce promoter activation over and above that seen in control cells.

Effects of C256Y and P301S mutations on differentiation of 3T3-L1 preadipocytes

We next determined the effects of WNT10B C256Y and P301S on adipocyte differentiation. Stable expression of wild-type *WNT10B* completely blocked the differentiation of 3T3L1 preadipocytes (Fig. 2b) by inhibiting expression of the adipogenic transcription factors

Cebpa and *Pparg* (Fig. 2d) as previously reported [5]. Similarly, WNT10B P301S cells failed to express *Cebpa* and *Pparg* and differentiate. In contrast, 3T3L1 preadipocytes constitutively producing WNT10B C256Y differentiated and accumulated lipid to the same extent as controls. Accordingly, the level of expression of *Cepba* and *Pparg* was indistinguishable between C256Y and control cells 6 days after the onset of differentiation. Thus, substitution of the highly conserved cysteine 256 for tyrosine results in loss of WNT10B function, abrogating its ability to activate canonical WNT signalling and block the differentiation of 3T3-L1 cells.

Discussion

This study is, to our knowledge, the first evaluation of *WNT10B* as a candidate gene for human obesity and reports the first non-synonymous variants of this gene. It also clearly demonstrates that at least one of these variants has severe functional impairment. The study used a mutational scanning design, and we did not examine whether common variants in or around this locus are associated with obesity or obesity-related phenotypes. Given the potential importance of WNT10B as a regulator of adipocyte differentiation, this should certainly be a priority.

How certain can we be that any of the variants we detected actually caused the obesity seen in the probands? In cases of rare missense variants, cosegregation with the phenotype of interest in large extended pedigrees combined with functional information on the adverse effects of the mutation are the gold standard for proving causality. Unfortunately, in most cases we did not have extensive family material available, and where we did the high prevalence of obesity in the background population makes it hard to formulate definitive statements about cosegregation. Having said this, the variant for which the best evidence is available is C256Y. The mutation disrupts an absolutely invariant cysteine residue, rendering WNT10B unable to activate WNT signalling and inhibit adipogenesis when stably expressed in a preadipocyte cell line. C256Y is likely to behave as a loss-of-function rather than a dominant negative mutation as we did not observe enhanced adipogenesis in our C256Y cell line compared with the control. The equivalent mutation in *Drosophila* wingless led to a molecule with impaired transport and signalling properties, resulting in abnormal epidermal patterning [17]. While some obese relatives of the affected proband were wild-type for *WNT10B*, all carriers of the variant were either overweight or obese. Also, one overweight carrier had almost twice the predicted percentage of body fat, suggesting that excess energy is preferentially partitioned into adipose tissue in this subject.

In addition to being lean, *Fabp4–Wnt10b* mice also have increased trabecular bone mass [7]. Conversely, *Wnt10b* knockout mice have decreased trabecular bone [18]; their adipose phenotype has yet to be reported. Although all carriers of the C256Y mutation have normal BMD, it is interesting that the bone mineral content of the proband with early-onset obesity is reduced (data not shown).

The fact that we found P301S in three unrelated UK children of European origin who had severe obesity and that it was absent in 100 UK and 200 Italian control subjects of European origin is notable. However, P301S retains autocrine function and the family information was

not strongly supportive of cosegregation. Although P301S was also active in paracrine assays (unpublished data, C. Christodoulides and A. Vidal-Puig), it remains possible that this variant influences susceptibility to adiposity through subtle changes in WNT10B function, which cannot be detected using in vitro overexpression assays.

What of the two variants found in the Italian obese subjects? Members of this group were less severely obese and their obesity was not selected to be of early onset; therefore, it is a priori somewhat less likely that monogenic or oligogenic factors will be found in this cohort. Two non-synonymous variants were found, neither of which results in substitution of a conserved amino acid residue. In one case (I285T), the limited family material did not allow any meaningful statements to be made about cosegregation, and in the other case (H77Y) one normal-weight sibling carried the variant and a homozygous parent was only mildly affected.

In summary, we established that non-synonymous variants of *WNT10B* exist in the human population and that at least one of these results in a loss of protein function that prevents its ability to block adipogenesis. The relationship of such variants to obesity is insufficiently established, but our results suggest that WNT10B might be a potential monogenic/oligogenic factor in severe familial obesity. This should encourage further study of this gene in other obese populations.

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Abbreviations

CEBPA	CCAAT/enhancer binding protein α
DHPLC	denaturing HPLC
LEF/TCF	lymphoid enhancer-binding factor/T-cell-specific transcription factor
LRP5/6	low-density lipoprotein receptor-related proteins 5 and 6
PPARG	peroxisome proliferator activated receptor γ
WNTs	wingless-type MMTV integration site family members
WNT10B	wingless-type MMTV integration site family, member 10B

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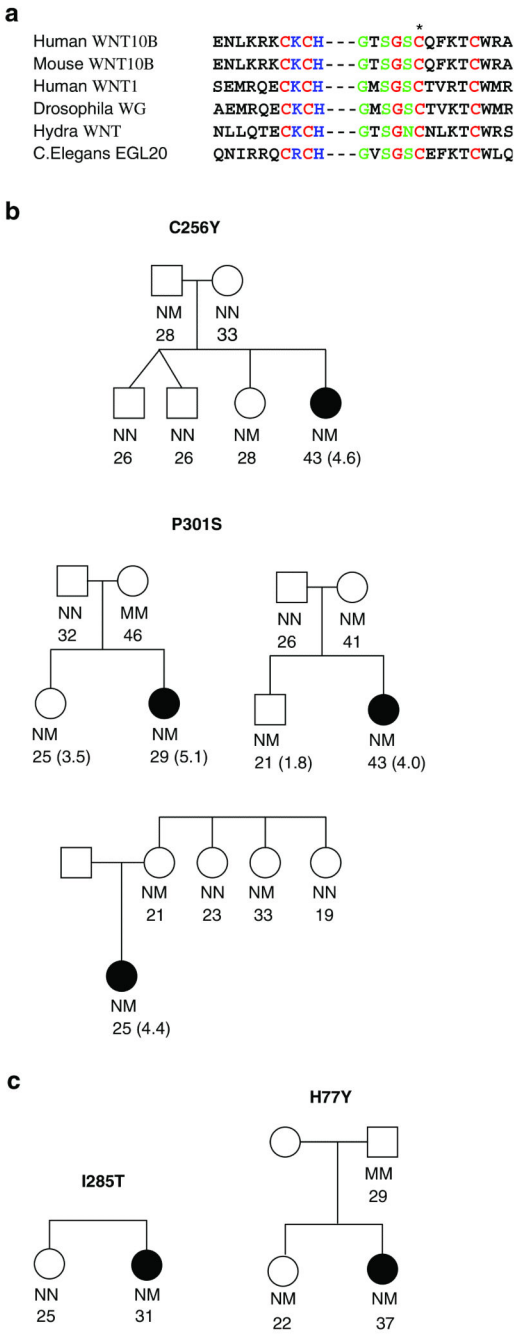

Fig. 1.

a Cysteine 256 is absolutely conserved among all WNT family members. **b** Family trees of all subjects with non-synonymous variants identified in group A (*early-onset obesity*) with BMI (BMI standard deviation scores for children) indicated. Probands are indicated by *filled symbols*. **c** Family trees of all subjects with non-synonymous variants identified in group B (*obese Italian subjects*) with BMI indicated. Probands are indicated by *filled symbols. M* variants on one allele, *N* normal genotype on one allele

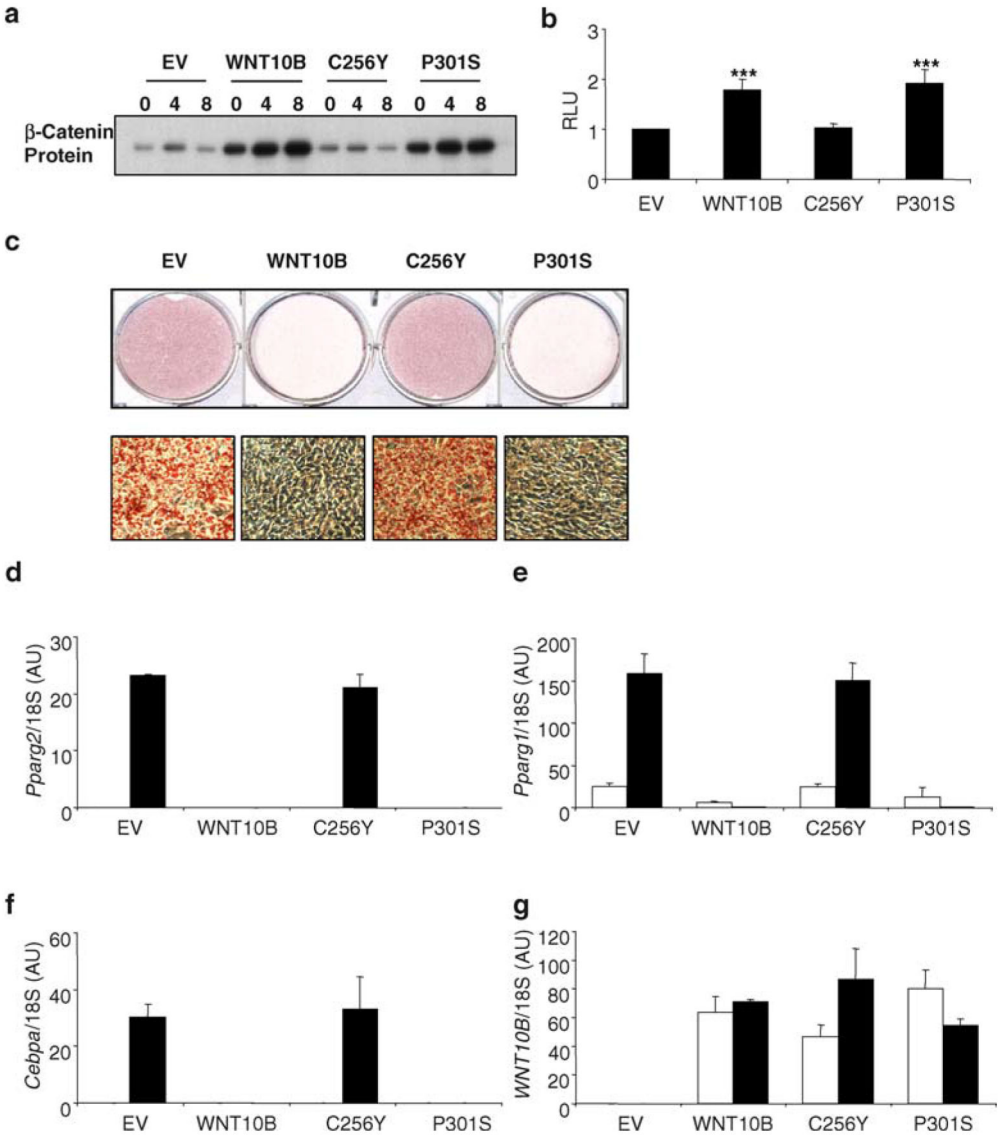

Fig. 2.

Functional properties of wild-type, C256Y and P301S WNT10B in cultured 3T3-L1 cells. **a** Western blot analysis of cytosolic β-catenin levels during differentiation of empty vector control (*EV*) and wild-type, C256Y and P301S WNT10B producing 3T3-L1 preadipocytes. *0* onset of differentiation (2 days after confluence); *4*, *8*, 4 and 8 days after induction of differentiation, respectively. Data are representative of three independent experiments. **b** Effect of wild-type, C256Y and P301S WNT10B on TOP-Flash reporter activity in 3T3-L1 cells. Results are expressed as fold difference relative to EV. All results are mean±SD of three independent experiments. ***$p<0.001$ for comparisons with EV. *RLU* relative luciferase units. **c** Effects of wild-type, C256Y and P301S WNT10B on differentiation of 3T3-L1 preadipocytes. *Top* Oil-Red O staining of 3T3-L1 cells 8 days after induction of differentiation. *Bottom* Light microscope images of cells stained with Oil Red O. **d–g** Expression of adipogenic markers and *WNT10B* as indicated in EV and wild-type, C256Y

and P301S WNT10B-producing 3T3-L1 cells at onset (2 days after confluence, *open bars*) and 6 days (*closed bars*) after induction of differentiation. All data are mean±SD of three independent experiments

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Table 1

Genetic data for the two cohorts

	Nucleotide change[a]	Coding change[b]	Type of change	Allele frequency
Cohort A	G767A	C256Y	Non-synonymous	Obese 1/192 Control 0/200
	C901T	P301S	Non-synonymous	Obese 3/192 Control 0/200
Cohort B	C229T	H77Y	Non-synonymous	Obese 1/230 Control 0/400
	T854C	I285T	Non-synonymous	Obese 1/230 Control 0/400
	C1059T	H353H	Synonymous	Obese 1/230 Control 0/400

Cohort A 96 early-onset obese subjects; *Cohort B* 115 obese Italian subjects; *Controls* 100 UK and 200 Italian subjects with normal weight

[a] Nucleotide position from transcription start site at which the sequence variant occurs

[b] Predicted amino acid substitution