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METHODS

Prevalence and Functionality of Paucimorphic and Private *MC4R* Mutations in a Large, Unselected European British Population, Scanned by meltMADGE

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Identification of unknown mutations has remained laborious, expensive, and only viable for studies of selected cases. Population-based "reference ranges" of rarer sequence diversity are not available. However, the research and diagnostic interpretation of sequence variants depends on such information. Additionally, this is the only way to determine prevalence of severe, moderate, and silent mutations and is also relevant to the development of screening programs. We previously described a system, meltMADGE, suitable for mutation scanning at the population level. Here we describe its application to a population-based study of *MC4R* (melanocortin 4 receptor) mutations, which are associated with obesity. We developed nine assays representing *MC4R* and examined a population sample of 1,100 subjects. Two "paucimorphisms" were identified (c.307G>A/p.Val103Ile in 27 subjects and c.–178A>C in 22 subjects). Neither exhibited any anthropometric effects, whereas there would have been >90% power to detect a body mass index (BMI) effect of 0.5 kg/m^2 at P = 0.01. Two "private" variants were also identified. c.335C>T/p.Thr112Met has been previously described and appears to be silent. A novel variant, c.260C>A/p.Ala87Asp, was observed in a subject with a BMI of 31.5 kg/m^2 (i.e., clinically obese) but not on direct assay of a further 3,525 subjects. This mutation was predicted to be deleterious and analysis using a cyclic AMP (cAMP) responsive luciferase reporter assay showed substantial loss of function of the mutant receptor. This population-based mutation scan of *MC4R* suggests that there is no severe *MC4R* mutation with high prevalence in the United Kingdom, but that obesity-causing *MC4R* mutation at 1 in 1,100 might represent one of the commonest autosomal dominant disorders in man. Hum Mutat 28(3), 294–302, 2007.

KEY WORDS: melanocortin 4 receptor; *MC4R*; mutation scanning; meltMADGE; paucimorphism; obesity

INTRODUCTION

The melanocortin-4 receptor (*MC4R*; MIM# 155541) is a G-protein-coupled seven-transmembrane receptor that is highly expressed in the brain and implicated in the central regulation of body weight [Cone, 2005]. MC4R is activated by α-melanocyte-stimulating hormone (MSH) [Fan et al., 1997], which is derived from proopiomelanocortin (POMC) and is antagonized by agouti-related protein (AgRP) [Ollmann et al., 1997]. Gantz et al. (1993) cloned *MC4R*, which encodes a 332–amino acid protein in a single exon localized on chromosome 18q22.

Several human studies have reported causality of early-onset severe morbid obesity by *MC4R* mutations leading to hyperphagia [Yeo et al., 1998; Vaisse et al., 1998; Hinney et al., 1999; Farooqi et al., 2000]. Based on the frequencies of mutations found in some case collections, it has been suggested that *MC4R* mutations

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constitute the most common monogenic form of human obesity, representing about 5% of severe human obesity [Farooqi et al., 2000, 2003].

Mutation searches of the *MC4R* gene have been performed by several groups based on small case collection samples of severely obese subjects and some lean control individuals. Only population studies can fully define the wide spectrum of mutational effects in *MC4R* covering severe, plus possible moderate, silent, or protective effects, and the full spectrum of polymorphism, paucimorphism (arbitrarily alleles $0.0005 < q < 0.05$ [Day et al., 2004]), and "private" mutations that may exist.

At present, there are several methods available for rapid mutation scanning [Cotton, 1997, 1998]. A major limitation is in the throughput for population scanning in the current methods. No technique has been available for scanning a sufficient number of nucleotides in unselected population samples or minimally selected case collections. Therefore, the extent of such variation at the population level and its impact on common traits has not been fully evaluated. The prevailing hypothesis for the molecular basis of common diseases is that it involves the combined actions of common polymorphic alleles of minor effect (common disease/common variant, CD/CV hypothesis). A contrasting approach has been the study of very highly selected cases and families by linkage and mutation detection techniques to identify rare mutations of a gene with large effect, often private to a single family (rare disease/rare variant, RD/RV hypothesis). However, intermediate possibilities exist. Sequence changes at an intermediate frequency may exist and may have a moderate effect [Day et al., 2004]. A number of different loci may predispose to the same disease, although only one paucimorphic allele of one particular gene might be important in any one individual. It is also possible that large numbers of "private" mutations of moderate effect could cumulatively account for a significant fraction of disease in a population. Exploring these hypotheses requires mutation detection in large numbers of relatively unselected cases and in representative population samples.

MeltMADGE combines the properties of MADGE [Day and Humphries, 1994; Gaunt et al., 2003] with a reconfiguration of denaturing gradient gel electrophoresis (DGGE) [Fischer and Lerman, 1979], using a thermal ramp in time [Gelfi et al., 1994], rather than a linear gradient in space, to increase the sample parallelism and reduce the costs of mutation scanning by one to two orders of magnitude. This methodology makes population scanning achievable. In previous tests (including blinded tests), 36 out of 37 different sequence variations in a wide variety of different sequence contexts, including mostly single-base changes but also a few small indels, were recognized [Alharbi et al., 2005]. Here we describe the development of a set of nine meltMADGE mutation scanning assays for the *MC4R* coding region and promoter and their application in a survey of a cohort of 1,100 anthropometrically characterized subjects.

MATERIALS AND METHODS

Subjects

MC4R (GenBank S77415.1) mutation scanning was performed using the North and East Hertfordshire cohort (HERTS). This comprises a group of healthy people born between 1920 and 1930 in the county of Hertfordshire (United Kingdom) and still living there at the time of follow-up 60–70 years later [Syddall et al., 2005]. A total of 660 men and 440 women (grand total 1,100 people) who had been traced consented for study following ethical approval. Genomic DNA was extracted from nucleated white blood cells in whole blood as previously described [Miller et al., 1988]. Birth-weight and 1-year weight were retrieved from original records and adult anthropometric traits, and other measures of cardiovascular health were taken in later adult life [Barker et al., 1992].

The prevalence study for the p.Ala87Asp mutation was carried out in the British Women's Heart and Health Study cohort (BWHSS). This is comprised of women recruited from 23 towns in England, Wales, and Scotland. In total, 3,525 samples were successfully genotyped from a total of 3,894 subjects. Full details of the selection of study participants and assessment of cardiovascular risk phenotypes have been previously published [Lawlor et al., 2003].

For HERTS, 18.5% of the cohort displayed a body mass index (BMI) $> 30\,kg/m^2$, while for BWHSS it was 26.5%.

DNA Amplification

MC4R gene-spanning PCR. "Long" PCR was used to minimise the use of bank DNA. A 3-kbp amplicon spanned the *MC4R* promoter and exonic region (Supplementary Fig. S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Primer sequences were: 5′-TTGCTGCCATCT TTCAAACCACCTTA-3′ (MRP5′) and 5′-CACGCAAAGT GTCCAATGCTTATGAA-3′ (MRP3′). Reactions of 20 µL contained 20 ng gDNA, 0.5 units/µL of *Pwo* DNA polymerase (supplied by Roche Diagnostics; www.roche-diagnostics.com), 0.5 units/µL *Taq* DNA polymerase, 1.0 mM Tris-HCl pH 8.3, and 5.0 mM KCl (all supplied by Promega; www.promega.com), 1.0 mM deoxynucleotide triphosphates (dNTPs), 5.0 mM $MgCl_2$, 1.0 pmol/µL primers (from MWG-Biotech; www.mwgbiotech.com), and 5 mM betaine (supplied by Sigma; www.sigmaaldrich.com). Thermal cycling consisted of 37 cycles: one cycle at 94°C for 2 min; 35 cycles at 94°C for 20 sec, 65°C for 30 sec, and 68°C 3 min; and one cycle at 68°C for 20 min.

Nested PCR reactions. PCR reactions representing regions of *MC4R* exon and promoter (Supplementary Fig. S1) were performed in 20 µL containing 1.5 mM $MgCl_2$ (Promega), 50 µM dNTPs, 10 mM Tris-HCl pH8.3, 50 mM KCl, 0.01% (wt/vol) gelatin, and 1.0 unit of *Taq* polymerase (all supplied by Promega), with 2 µL of 1 to 100 dilution in water of long PCR template added to each well and 1 pmol of each primer (Supplementary Table S1). Thermal cycling consisted of an initial denaturant temperature step at 96°C for 5 min, followed by 30 cycles with denaturing step for 30 sec at 96°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C. A final extension step at 72°C for 2 min was used.

Allele-specific PCR. Amplification refractory mutation system (ARMS) assays were designed to confirm findings following sequencing of variants (p.Val103Ile, p.Thr112Met, and p.Ala87Asp) identified by meltMADGE and were used to rescreen the long PCR DNA bank. A total of 2 µL of 1 to 100 dilution of long PCR was used as template in a 10-µL ARMS PCR reaction. The reaction mix contained: 1 µL of 10 × PCR buffer, 1 µL of 15 mM $MgCl_2$, 0.2 µL of primer mix (2 µL of forward primer, 10 µL of reverse, and either of the allele specific primers, all at concentration of 100 pmol/µL) (Supplementary Table S2). A total of 1.0 unit *Taq* polymerase and 7.3 µL of deionised water (dH_2O) was used. Thermal cycling consisted of 37 cycles: one cycle at 94°C for 2 min; 35 cycles at 94°C for 30 sec; 50–60°C for 30 sec; and 70°C for 30 sec; and one cycle at 70°C for 2 min.

meltMADGE

A detailed description of the meltMADGE methodology is available [Alharbi et al., 2005; Smith et al., 2006]. MADGE

formers and glass plates of the same dimensions were used essentially as previously described [Day and Humphries, 1994; Gaunt et al., 2003], except that meltMADGE gels as used in this work contained 6 M urea. Approximately 2 μL of PCR product was loaded from microplates by passive transfer using a 96-slot pin replicator onto the meltMADGE gel. Prior to loading into the gel tank each gel was sealed as previously described [Alharbi et al., 2005; Smith et al., 2006]. Electrophoresis was in 1 × tris-acetate (TAE) buffer of the same ionic strength and with equivalent ammonium persulfate (APS) concentration as found in the gels to prevent spatial ionic heterogeneity. Electrophoresis was for 2–3 hr at 50 V and 2 A, with a linear ramp temperature. For temperature ramps of *MC4R* amplicons see Supplementary Table S1.

Gels were stained in 100 mL of 1 × TAE buffer with 10 μL of Vistra Green (Molecular Probes; www.invitrogen.com) and visualized using a FluorImager 595 (GE Healthcare, Amersham Biosciences; www5.amershambiosciences.com), as described [Gaunt et al., 2003].

Generation of Artificial Positive Controls

Artificial positive controls were generated by synthesis of amplicons using primers (Supplementary Table S1) with a one-base chemical mutation (at position –4 from 3′ end). and coannealing with a similar quantity of wild-type (WT) amplicon (synthesized using primers perfectly matched to the genomic template). These were used to generate heteroduplexes as previously described [Smith et al., 2006].

Direct Sequencing

DNA sequencing was performed for the samples that showed meltMADGE patterns different from WT using Big Dye Terminator cycle sequencing (Applied Biosystems, Foster City, CA; www.appliedbiosystems.com) reading reaction and products resolved by ABI PRISM 377 DNA sequencer (Applied Biosystems). Residual PCR product from meltMADGE assays was used as template, sequencing primers were 5′-CTCTGGGTGTCATCAGCTTG-3′ forward and 5′-CAAGGAGCTACAGATCACCGA-3′ reverse for second fragments of exon 1, 5′-GAGAATGAATATTTTGAAG CC-3′ forward and 5′-AGGGACTCACTGGCATTGCTG-3′ reverse for fourth fragment of the promoter. Residual long PCR in the respective meltMADGE exon and promoter PCR products were used as template for sequencing reaction.

PolyPhen Analysis of Codon Changes

To assess the possible effect of amino acid substitutions, we used the Polymorphism Phenotyping (PolyPhen) program for automated functional annotation of nonsynonymous coding SNPs (nsSNPs) [Ramensky et al., 2002]; available on the web (http://tux.embl-heidelberg.de/ramensky).

Studies of Mutant Receptor Function

Using methods described in detail previously [Farooqi et al., 2003], WT (normal) and mutant *MC4R*s were cloned into the mammalian expression vector pcDNA3 (Invitrogen, Paisley, UK; www.invitrogen.com) and transiently transfected into HEK293 cells with a luciferase reporter under the control of a promoter that was responsive to cyclic AMP (cAMP) according to the manufacturer's protocols (Fugene Reagent, Roche Diagnostics). All transfections incorporated a pRL-cytomegalovirus (CMV) plasmid (Promega), which constitutively expresses *Renilla* luciferase and controls for the efficiency of transfection. Cells were deprived of serum, and various concentrations of α-melanocyte-stimulating hormone (Bachem, Weil am Rhein, Germany; www.bachem.com) were added. After the cells were harvested, luciferase activity was determined with a luciferase assay system (Promega). Activation of MC4R increases intracellular cAMP, which stimulates the expression of luciferase. Each experiment was conducted in quadruplicate; values are given as means ± SE. Data were analyzed and curves were fitted with the use of Origin software (OriginLab; www.originlab.com).

Direct Assay for *MC4R* p.Ala87Asp

A LightTyper (Roche Applied Science; www.roche-applied-science.com) assay was designed to allow the rapid screening of the BWHSS cohort for the c.260C > A sequence variation based upon a dual probe Dabcyl-quenching assay [Gaunt et al., 2006]. All oligonucleotides were from MWG-Biotech. These were: MC4Rp.Ala87Asp-F 5′-GAGAATATCTTAGTGATTGTGGCA, MC4Rp.Ala87Asp-R 5′-TTGAAACGCTCACCAGCA, Dabcyl Anchor Probe 5′-TGCATTCACCCATGTACTTTTTCATC-Dabcyl-3′, and an allele-specific fluorescein isothiocyanate (FITC) Probe 5′Flu-CAGCTTGG(C)TGTGGC-Phosphate-3′ (c.260C > A site in FITC probe in (C)). A 109-bp region of the MC4R gene surrounding the base variation site was PCR amplified asymmetrically using the forward and reverse primers on a PTC-225 thermocycler (MJ Research; www.mjr.com). Each 10-μL reaction contained the manufacturer's reaction buffer, genomic template, 0.1 μM of MC4RP.ALA87ASP-F, 0.5 μM of MC4RP.Ala87Asp-R, 0.2 μM Dabcyl probe, 0.1 μM FITC probe, 1.5 mM $MgCl_2$, 0.2 μM dNTPS, and 0.025 units/μL Taq polymerase (Promega). Amplification conditions involved an initial 2 min denaturation at 95°C then 69 cycles of 95°C for 20 sec, 55.4°C for 25 sec, 72°C 20 for sec, and a final elongation step at 72°C for 5 min. Prior to screening using the LightTyper, the samples were overlaid with 10 μL Chill Out Wax (MJResearch). The samples were subjected to a melting ramp of 0.1°C/sec and the fluorescence measured over a 30°C–70°C temperature range.

RESULTS

Validation of MC4R meltMADGE Assays and Use of MC4R Long PCR "Bank"

Each of the nine amplicons was designed with GC-clamps such that the predicted temperature of melting was essentially the same along the length of the amplicon, which should ensure detection of most of the possible sequence changes. WT amplicon was confirmed to show major mobility transition (fast to slow) at a temperature near the upper end of the thermal ramp range used. WT amplicons each showed a pattern of one band whereas all artificial positive control amplicons showed split-band patterns (Supplementary Fig. S2). It was confirmed (using the known p.Val103Ile paucimorphism) that both meltMADGE assays and direct sequencing of meltMADGE amplicon gave "clean" results (no extra bands or strong background) without allelic bias when a long PCR representing *MC4R* was used as template, compared with using genomic DNA as template (data not shown). Therefore, a long PCR bank derived from the stock genomic DNA bank (a finite resource) was used to avoid repeated access of stock genomic DNA for each of the nine meltMADGE assay amplifications. For p.Val103Ile, p.Thr112Met, and p.Ala87Asp identified in meltMADGE assays, direct ARMS tests (data not shown) were constructed and applied to the *MC4R* long PCR bank in order to confirm that meltMADGE assay had detected all of these specific sequence variants present in the 12 96-well arrays.

Sequence Changes Identified in HERTS Cohort by meltMADGE Mutation Scanning

A total of 52 subjects from the HERTS cohort were identified displaying band patterns different from WT, in each case suggestive of heterozygosity for a single-base sequence change (resolved rather than coelectrophoresing heteroduplexes). These included 22 subjects with an apparently identical variant pattern in amplicon MRP4 and 30 subjects with three different variant band patterns in amplicon MR2. All were characterized by direct sequencing (Figs. 1 and 2) as summarized in Table 1 and described below.

c.–178A>C (5′ Untranslated Region)

Upon direct sequencing (Fig. 1), all 22 subjects (out of 1,071 yielding PCR products) with variant patterns in MRP4 were shown to be heterozygous for c.–178A>C (for cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon; GenBank S77415.1.). This previously described [Jacobson et al., 2002] transversion is located 178 nucleotides upstream of the initiator methionine codon, in the 5′ untranslated region (UTR) of *MC4R* mRNA. The WT A base represents the first in a string of four As, in a generally A-rich region. No significant associations were found between c.–178A>C and late-life or early-life

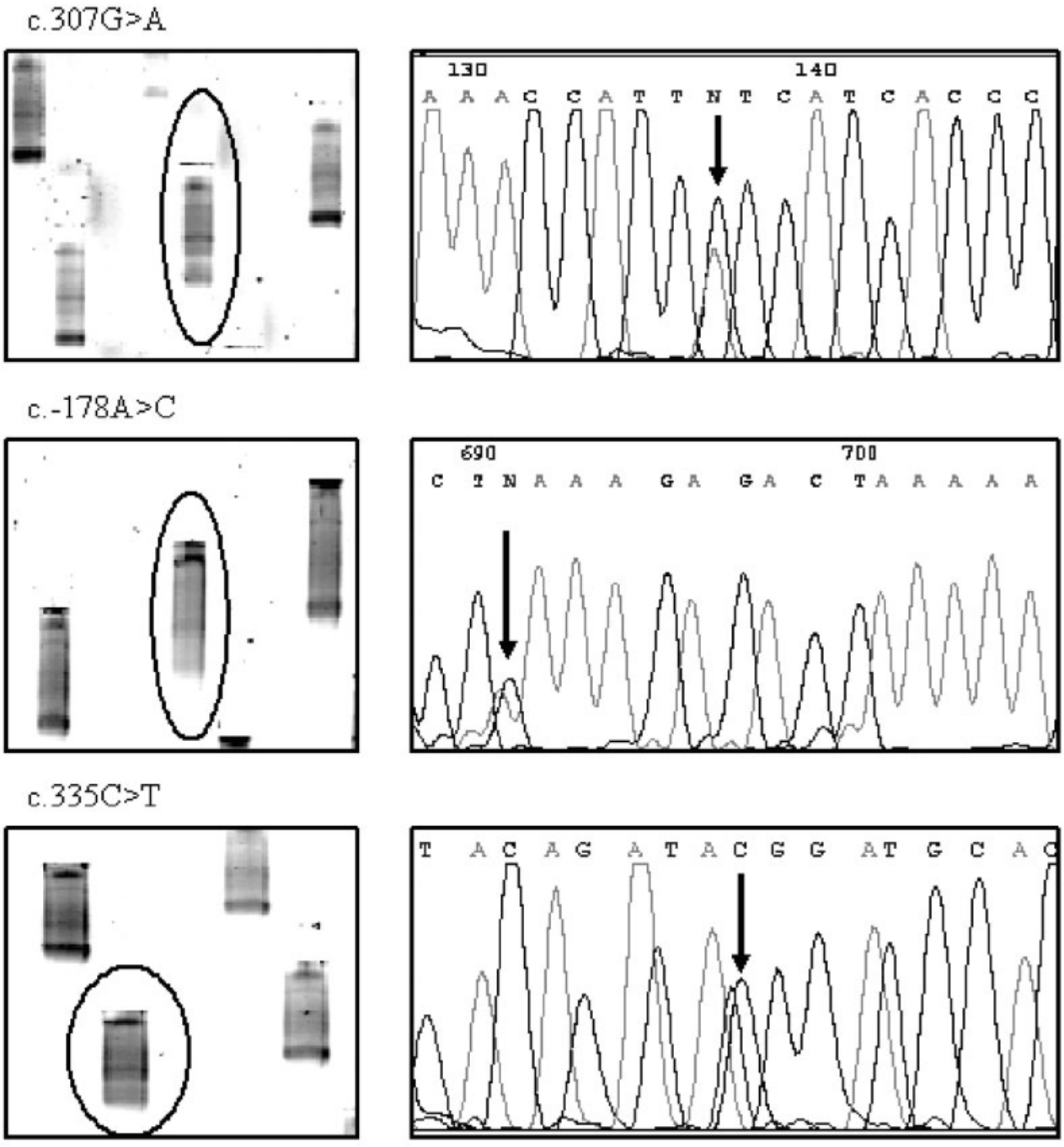

FIGURE 1. **meltMADGE patterns and sequencing confirmation of *MC4R* mutations found in the HERTS cohort for previously reported mutations in *MC4R*.**

anthropometric phenotypes for men, women, or men and women combined (Table 2).

c.260C>A (p.Ala87Asp)

One mutation (c.260C>A), which has not been described previously, was found in region MR2 of *MC4R*. In Figure 2, we show an overview of this cohort scan, embodying 12 arrays scanned in two tank runs (Fig. 2A), with focus on one array containing 1 out of 96 tracks with a split band pattern (Fig. 2B) and close up of this 2.6-cm track and subsequent sequencing (Fig. 2C). The mutation was observed in a single subject (1/1,089) at a CpG site and leads to change of an encoded alanine, which is nonpolar, to aspartic acid, a charged and polar amino acid. The PolyPhen program score was 2.199 (Table 3); scores ≥ 1.5 are predicted to be possibly damaging. The signaling properties of this mutant receptor were studied using a cAMP responsive luciferase reporter assay as previously described [Farooqi et al., 2003]. p.Ala87Asp results in a partial loss of function in vitro (Fig. 3). The BMI for this subject was 31.5 kg/m^2, which fulfils the definition of clinical obesity (Table 2). A LightTyper assay was developed to enable the prevalence of this mutation to be tested using a larger sample set not based around a single geographical location. Direct assay for p.Ala87Asp was clearly positive for the subject identified (HERTS), but was negative in a further 3,525 subjects for whom PCR amplification for genotyping was successful from the BWHHS cohort (Fig. 4).

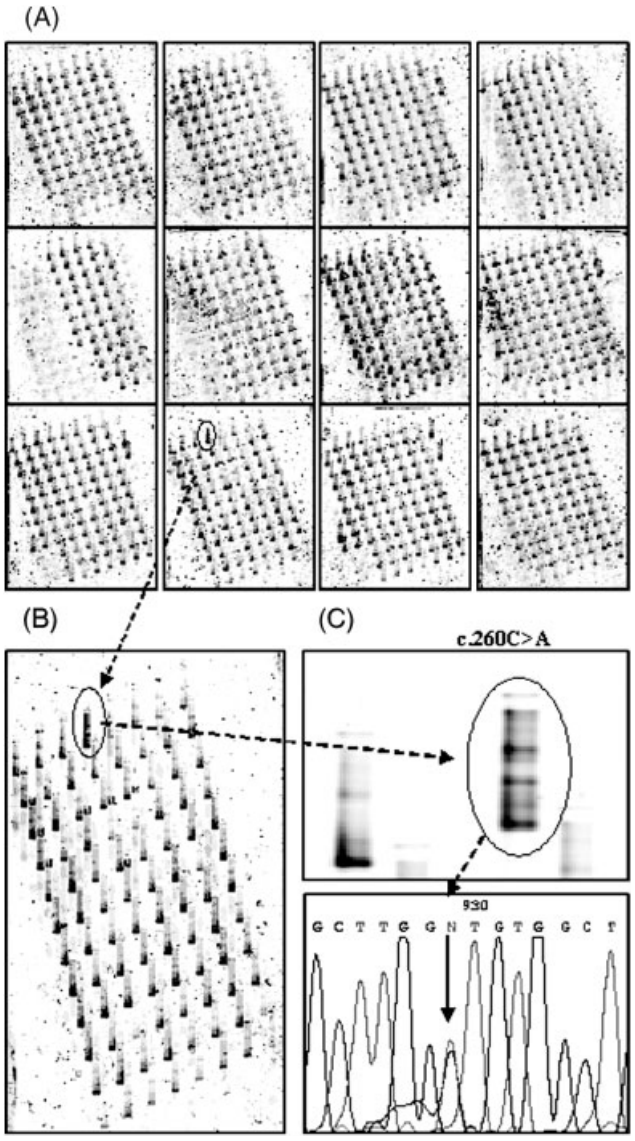

FIGURE 2. **A: Overview of one run (amplicon MR2) of 12 gels (1,100 subjects). Focus on the array containing c.260C>A mutation, highlighted by marked ring (B) magnification of the band pattern and subsequent sequencing to confirm mutation identity (C).**

c.307G>A (p.Val103Ile)

Of 1,089 (HERTS) subjects, 28 showed an apparently identical variant meltMADGE band pattern in amplicon MR2, an example of which can be seen in Figure 1. On sequencing, all exhibited heterozygosity for c.307G>A. These were reconfirmed via ARMS assay. This transition has been reported previously [Gotoda et al., 1997] and leads to valine to isoleucine substitution at codon 103 (p.Val103Ile), but does not impair receptor signaling through cAMP in vitro [Farooqi et al., 2000]. The PolyPhen score was 0.858 (Table 3). Mean BMI of carriers was 26.95 kg/m^2, not significantly different from the WT of 26.82 kg/m^2 ($P = 0.86$). Birth weight and 1-year weights were also essentially identical with the WT (Table 2).

c.335C>T (p.Thr112Met)

One subject (1/1,089) with a unique variant meltMADGE band pattern (Fig. 1) in amplicon MR2 was found to have the c.335C>T mutation previously reported [Gu et al., 1999]. A specific ARMS-MADGE assay did not identify any other carriers in the *MC4R* long PCR bank. The base change is at a CpG site and leads to change from threonine, which is polar, to methionine, a nonpolar amino acid. The subject had a BMI of 24.05 kg/m^2. The PolyPhen score was 1.021 (Table 3). This mutation has previously been shown to result in normal receptor signaling in vitro [Gu et al., 1999].

DISCUSSION

The ability to undertake mutation scanning of *MC4R* at the level of the "whole" population was enabled in our laboratory by the development and application of meltMADGE methodology.

TABLE 1. **Summary of Findings in *MC4R* Using meltMADGE***

MC4R	Nucleotide change	Amino acid change	Reference	Remarks
5′UTR	c.−178A>C	5′UTR	Jacobson et al. [2002]	22 subjects
Exon 1	c.307G>A	p.Val103Ile	Gotoda et al. [1997]	28 subjects
	c.335C>T	p.Thr112Met	Gu et al. [1999]	One subject, BMI 24.1
	c.260C>A	p.Ala87Asp	New	One subject, BMI 31.5; possible functional significance

*The MC4R cDNA sequence (Genbank Acc. No. S77415.1) is numbered +1 starting with the A of the ATG translation initiation codon.
BMI, body mass index; UTR, untranslated region.

TABLE 2. Anthropometric Characteristics for Two Paucimorphisms and Two "Private" Mutations in *MC4R*

	N	N	BMI(kg/m^2) WT		BMI(kg/m^2) HET			Birth weight (oz.) WT		Birth weight (oz.) HET			Weight at 1 year (oz.) WT		Weight at 1 year (oz.) HET		
Mutation	WT	HET	Mean	SD	Mean	SD	P	Mean	SD	Mean	SD	P	Mean	SD	Mean	SD	P
c.–178A>C	1049	22	26.90	3.86	26.76	4.47	0.86	123.0	18.4	128.4	16.3	0.21	353.0	41.0	361.1	40.7	0.46
p.Val103Ile	1061	28	26.95	3.87	26.82	3.64	0.86	123.0	18.5	124.4	19.4	0.69	353.5	41.4	361.4	28.0	0.32
p.Ala87Asp	–	1			31.50					168.0			392.0				
p.Thr112Met	–	1			24.05					92.0			344.0				

WT, wild-type; HET, heterozygote; N, number of subjects; P, probability value; SD, standard deviation.

TABLE 3. Predictions for *MC4R* Amino Acid Substitutions

Substitution in *MC4R*	PolyPhen score[a]	PolyPhen prediction	Type of substitution
p.Val103Ile	0.858	Benign	Conservative substitution
p.Thr112Met	1.021	Benign	Conservative substitution
p.Ala87Asp	2.199	Probably or possibly damaging	Substitution in the transmembrane region of a hydrophobic residue by an acidic residue

[a]Score ≥1.5 probably or possibly damaging (may have functional effect).

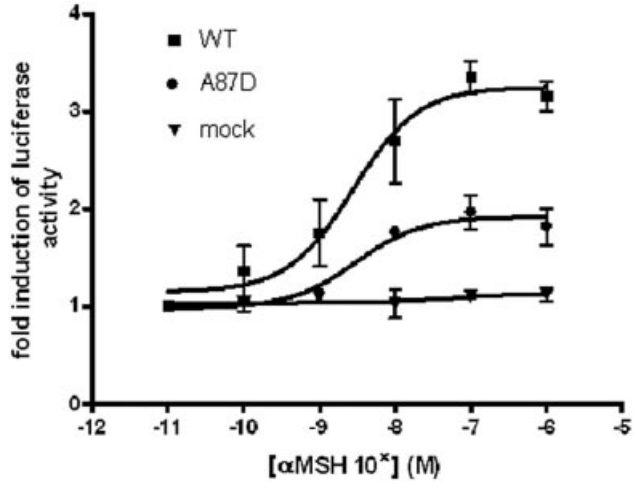

FIGURE 3. **MC4R (p.Ala87Asp) has markedly reduced ability to stimulate production of cAMP as assessed by cAMP-responsive luciferase reporter assay. Graph indicate responses of WT, mutant (p.Ala87Asp), and mock-transfected cells to a logarithmic increase in alphaMSH concentration. Each point represents the mean (±SEM) of three independent experiments performed in quadruplicate.**

With appropriately configured assays, the approach is sensitive to most single-base changes, relies on minimal capital expenditure and per subject-amplicon costs of one-seventh rather than 30 times the PCR cost [Alharbi et al., 2005]. Unbiased insight into the prevalence and effects of rarer sequence variation can be derived from unselected subjects, whereas previous studies have mainly focused on mutation scanning in highly selected clinical cases. Nine amplicons representing *MC4R* were scanned in 1,100 subjects at a total cost of 1,000 Euros in 2 weeks.

Comments on the meltMADGE Methodology

Assay design was simplified using an in-house program, Tixis, but several such assay design utilities configured around the original melt87 [Lerman and Silverstein, 1987] and melt94 algorithms are available. Critical parameters in the assay are accuracy and precision of temperature control and well-calibrated equipment. Furthermore external sensors regularly calibrated to national standards will ensure portability of assays between equipment and laboratories. For the overlapping amplicons planned to cover *MC4R*, flat predicted melting profiles were achieved and artificially (PCR) constructed mutation "heterozygotes" showed heteroduplex band resolution compared with the WT. Both previously described [Gotoda et al., 1997; Jacobson et al., 2002] paucimorphisms (c.–178A>C and c.307G>A) in *MC4R* were detected in amplicons MRP4 and MR2, respectively. We constructed ARMS assays [O'Dell et al., 2000] for three of the sequence variants identified. These ARMS tests did not identify any other mutation carriers other than those detected in various array positions by the meltMADGE assays. Last, dHPLC assays on several plates (data not shown) did not identify any other sequence variants other than those described above.

Epidemiological and population studies are costly and often the available DNA resources are finite: conservative usage is therefore essential. Long PCR was used in this study primarily to reduce consumption of genomic DNA. One long PCR from 20 ng genomic DNA covered the *MC4R* entirely at a 1 in 100 dilution; this then supported nine shorter amplicons for meltMADGE assays. This bank has also been retained for further potential studies. During initial analyses of p.Val103Ile, we confirmed both by meltMADGE and by sequencing that there was no allelic bias during long PCR amplification. An additional advantage is that the high yield of specific short amplicons and the additional cycles of polymerase activity (30 cycles for long PCR and 28 cycles for meltMADGE PCR) did not appear to compromise background (polymerase error heteroduplexes) for mutation scanning.

Specific Comments on Sequence Variants Identified

A 2-kb genomic segment representing the *MC4R* coding region and promoter was scanned in approximately 1,100 unselected subjects. Two paucimorphisms (c.–178A>C and p.Val103Ile) and two "private" sequence variants (p.Thr112Met and p.Ala87Asp) were identified. One of the "private" sequence variants (p.Ala87Asp) has not been described previously and may be of phenotypic relevance.

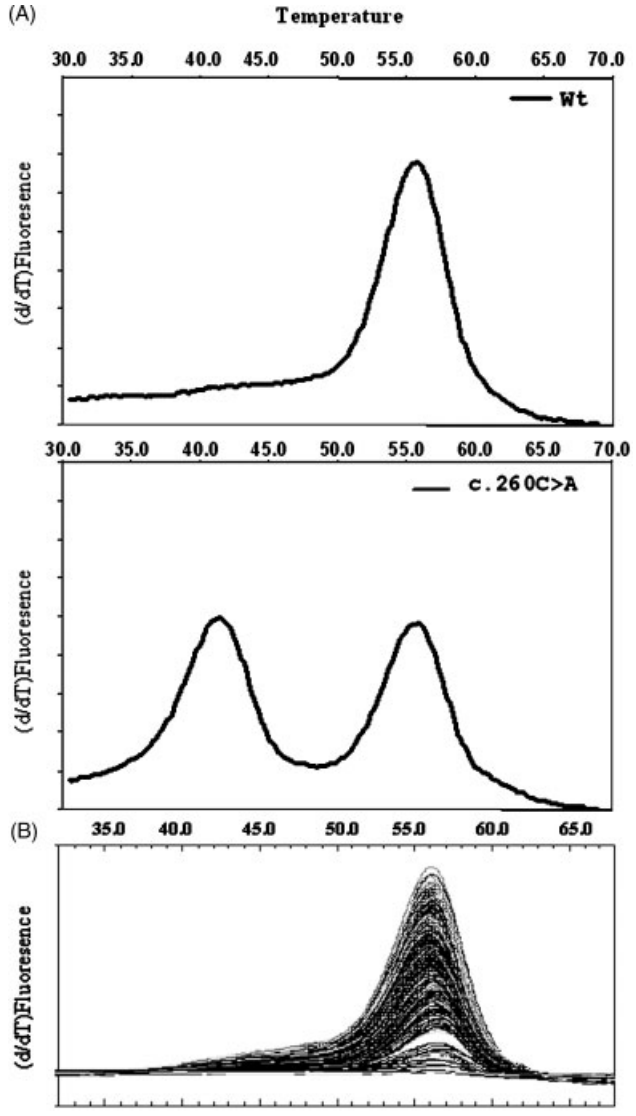

FIGURE 4. **LightTyper assay for p.Ala87Asp mutation. A: The array containing the single c.260C>A mutation was analyzed to validate the LightTyper assay. The FITC probe was designed to form a perfect match with the WT sequence. Homozygotes for the C allele produced a single melt curve, while the heterozygous c.260C>A mutation demonstrated a distinctive double melt curve corresponding to the variation in FITC probe melting caused by perfect binding to the WT sequence and mismatched binding to the mutant sequence. B: BWHSS 384-well plate p.Ala87Asp LightTyper genotyping assay, all positive samples demonstrated a single WT melting curve.**

c.–178A>C (5′UTR)

The 5′UTR sequence variant c.–178A>C (Fig. 1) was present at an allele frequency of 0.0101 (22/2178 alleles). This compares with estimated rare (C) allele frequencies of 0.021 in 216 "White" nonobese controls pooled from Sweden and the United States [Jacobson et al., 2002], and 0.073 in 48 "Black" nonobese controls [Jacobson et al., 2002]. Estimated allele frequencies in 1,089 × 2 "White" and "Blacks" [Jacobson et al., 2002] were 0.0325 and 0.117, respectively, which were higher than in the control groups but with no strong statistical evidence to support a difference other than due to chance. We found nonsignificantly lower mean BMI in the –178C heterozygote group (Table 2). While the 5′UTR of eukaryotic mRNA plays a crucial role in the regulation of gene expression, controlling mRNA localization, stability, and translational efficiency [Pesole et al., 2002], the c.–178A>C variant does not appear to have functional features. Upstream gene reading frames (ORF), represent a regulatory mechanism for eukaryotic protein synthesis [Meijer and Thomas, 2002] as do internal ribosome entry sites (IRES), at GC-rich regions [Stoneley and Willis, 2004], hairpins, other folding, and binding proteins. The *MC4R* 5′-UTR is 426 nt in length and contains several potential upstream methionine codons, although none with a strong Kozak consensus [Kozak, 1986] comparable with the initiator methionine. The c.–178A>C variant eliminates a potential TAA stop codon, which could be relevant to such an ORF, although there is another frame stop codon shortly 5′ to this site. The –178 site is in an A-rich region and its possible relevance to 5′UTR folding remaining obscure. At the population level, this variant appears to be functionally neutral in relation to body weight.

c.307G>A (p.Val103Ile)

Our estimated population allele frequency of p.Val103Ile was 0.0129. Meta-analysis based largely on case–control studies [Geller et al., 2004; Heid et al., 2005] summarized carrier frequencies in nonobese controls various studies ranging from 1.9 to 5.7% (i.e., allele frequencies 0.0095 to 0.0275), whereas allele frequencies in obese cases were lower, one allele giving an odds ratio of 0.69 for obesity (95% CI, 0.50–0.96). A more recent meta-analysis of 7,937 individuals also found a mild negative association with obesity [Heid et al., 2005]. Our population-based study found no significant mean difference (p.Val103Ile carrier BMI = 26.82 kg/m^2 vs. WT BMI = 26.95 kg/m^2; P = 0.86) in obesity for 103I carriers, nor distributional bias of BMI for p.Val103Ile carriers. There was over 90% power at P = 0.01 to detect an BMI difference of 0.5 units. Case–control data and meta-analysis remain susceptible to various biases in ascertainment, pooling, and publication. Rare allele frequencies are more likely than common alleles to show geographical variation, for example, and correlations of other nutritional, environmental, social, and even other genetic factors with geography could confound analyses.

p.Val103Ile represents a conservative substitution as indicated by PolyPhen analysis (Table 3) in *MC4R* transmembrane domain two and in vitro functional studies (Geller et al. [2004] and references therein) have not identified differences from the WT.

c.335C>T (p.Thr112Met)

p.Thr112Met has been observed previously in 1 out of 243 United Kingdom obese cases and 1 out of 108 controls [Farooqi et al., 2000], in 2 out of 306 German cases [Hinney et al., 1999], and in 2 out of 469 Swiss cases [Branson et al., 2003]. The subject identified in our (United Kingdom–based) population study was not obese (BMI = 24.05 kg/m^2). Although this substitution in the first extracellular loop of the receptor is not conservative, this mutation does not result in impaired MC4R signaling through cAMP in vitro [Gu et al., 1999]. It appears that p.Thr112Met is a rare, silent variant, widely distributed at least within Europe.

c.260C>A (p.Ala87Asp)

The subject bearing the previously unreported sequence change c.260C>A (pAla87Asp) fulfils the clinical definition of obesity (BMI ≥ 30 kg/m^2). In silico predictions (Table 3) indicated a deleterious mutation and expression assays showed that this mutation results in impaired signaling through MC4R in vitro. Hypothesizing that p.Ala87Asp might be a common or moderate

mutation in the general population, but possibly not so evident in extreme case ascertainments, we tested a further 3,525 subjects. Absence of p.Ala87Asp in these subjects, drawn from 23 towns in Britain, makes it very unlikely that p.Ala87Asp is either a prevalent severe or moderate obesity mutation in the United Kingdom.

Ethical permissions in anonymized cohort studies unfortunately preclude further family follow-up. The identification of this *MC4R* sequence variant on a population basis illustrates the potential to identify the contribution of severe or moderate (*forme fruste*) mutations to diseases at the population level.

Both of the rare mutations each identified in our individual were at CpG sites. In another study [Alharbi et al., 2005], 5 out of 6 sequence variants of the low density lipoprotein receptor gene identified on a population study were at CpG sites. Methyl-CpG sites are well known to be prone to rapid mutation to TpG (or CpA on the opposite strand). In contrast with case collections ascertained for very severe phenotypic effects (and hence for particular type of mutation including indels, frameshifts, etc.), it seems possible that CpG mutation might be even more prevalent than previously recognized and could be responsible for a great proportion of mutations with moderate or mild phenotypic effects. Such studies necessarily demand sequence scanning of large numbers of individuals in order to identify a few. Even in extreme obesity case collection, only a small proportion (0.5–5%) have been found to display *MC4R* mutations [Farooqi et al., 2000; Vaisse et al., 2000]. Nonetheless, although the confidence interval is wide, if 1 out of 1,100 is representative of the prevalence of obesity-causing mutations in the general population, then obesity due to *MC4R* deficiency represents one of the most common autosomal dominant disorders in man.

In this study, we have applied a new and cost-efficient mutation scanning approach to explore the spectrum of rare sequence variants at the population level. This has given insight into the phenotypic effects of two paucimorphisms, and although it has not identified any "protective" mutations causing thinness, it has given an initial estimate of severe disease causing mutational prevalence in *MC4R*.

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