Functional Characterization of Variants in *MC4R* Gene Promoter Region Found in Obese Children

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Context: Mutations in the *MC4R* gene are the most common cause of monogenic obesity, and there are few studies on mutations in the promoter region.

Objective: The objective of the study was to sequence the promoter region of the *MC4R* gene in a cohort of obese children to identify rare variants.

Design, Setting, and Patients: A region 1500 bp upstream of the *MC4R* gene was sequenced in 267 unrelated local children younger than 10 years, with body weight of at least 150% of ideal. An 891-bp upstream region of the *MC4R* gene was cloned into a luciferase reporter vector for reporter gene assays.

Interventions: There were no interventions.

Main Outcome Measures: The basal transcriptional activity of the *MC4R* promoter was analyzed in human embryonic kidney 293 cells using reporter gene assays.

Results: Three rare variants were detected: c.-803A>G, c.-105C>G, and c.-216C>T. The novel c.-803A>G variant was found in a 9-year-old severely obese Malay boy. This variant was not found in his severely obese mother but was present in his overweight father, who had type 2 diabetes, and also in his normal-weight brother. The novel c.-105C>G variant was found in an obese 9-year-old Malay boy. The c.-216C>T variant was found in an obese Chinese girl with Down's syndrome. The transcriptional activities of the c.-803A>G and c.-105C>G promoters were significantly reduced compared with the wild type but not the c.-216C>T promoter.

Conclusions: We have described, for the first time, two novel human *MC4R* gene promoter variants found in obese children that resulted in a decrease in basal transcriptional activity. **(*J Clin Endocrinol Metab* 99: E931–E935, 2014)**

Mutations in the *MC4R* gene constitute one of the most common causes of human monogenic obesity (1). *MC4R* mutation carriers have severe obesity, hyperphagia, hyperinsulinemia, increased lean mass, increased linear growth, and bone mineral density (1). *MC4R* mutations result in an autosomal codominant form of obesity with variable expressivity and incomplete penetrance (1, 2). Mutations in the *MC4R* gene include frame shift, nonsense, and missense mutations scattered throughout the coding region that impair receptor signaling through reduced cell surface expression, reduced ligand binding, or reduced downstream signaling (2–4).

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Abbreviations: BMI, body mass index; HbA1c, glycated hemoglobin A1c; LDL-C, low-density lipoprotein-cholesterol; MAF, minor allele frequency; MC4R, melanocortin-4 receptor; SNP, single-nucleotide polymorphism; TC, total cholesterol; WFH, weight for height.

A reduction in gene transcription caused by mutations in the *MC4R* promoter may potentially cause obesity in humans (5). Two putative transcription start sites have been identified in the human *MC4R* promoter at −139 bp and −426 bp from the translation start site (Figure 1A), and the minimal core promoter region was defined as −556 to −416 bp upstream of the *MC4R* translational start site (6). A region −1926 bp up to the translational start site has been shown to have effects on transcriptional activity (6–8). In this study, we screen the *MC4R* promoter region for variants that may be associated with early-onset obesity in our local population.

Subjects and Methods

Study subjects

The *MC4R* promoter region was analyzed in 267 unrelated children and adolescents with severe obesity having a body weight of at least 150% of the ideal weight for height (WFH) and onset of obesity before age 10 years. Fifty-one percent were Chi-

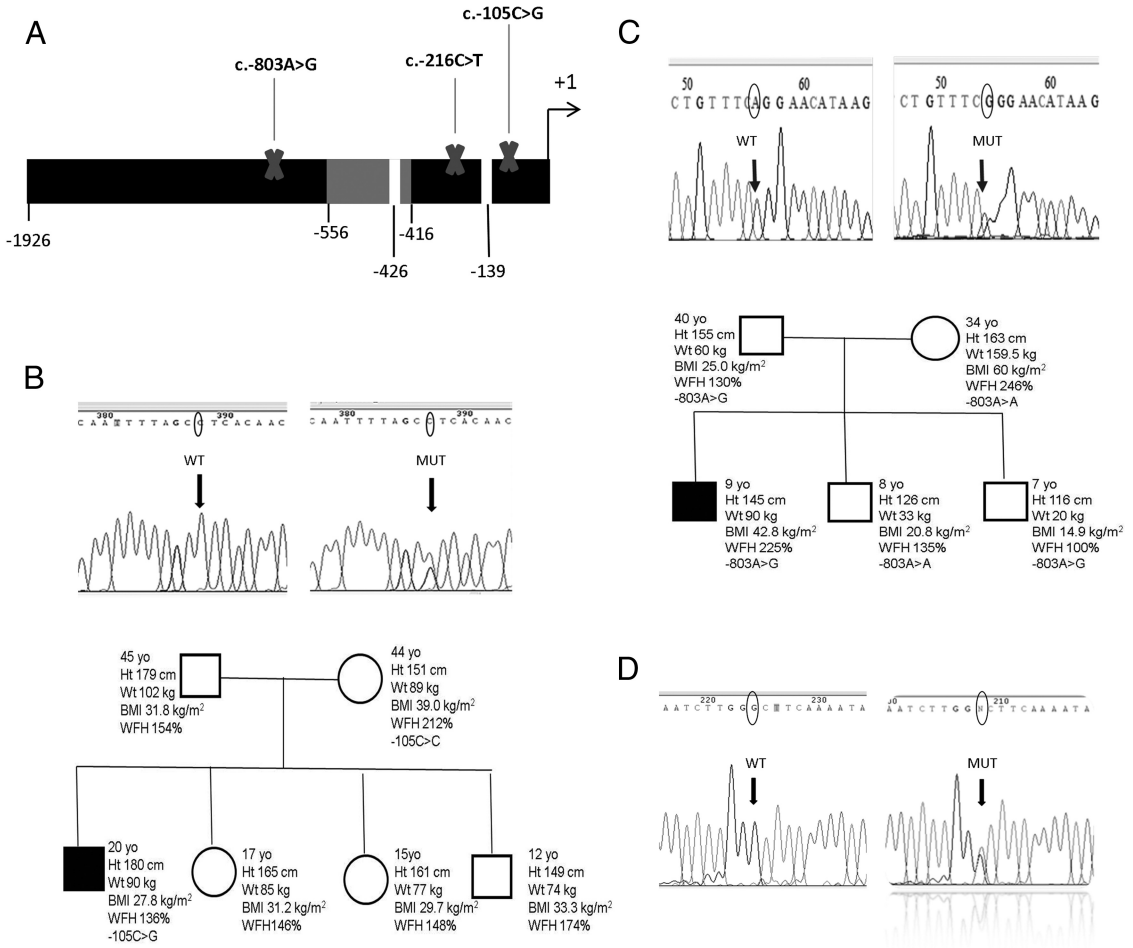

Figure 1. A, Promoter region of the *MC4R* gene. The putative transcription start sites at −426 and −139 are indicated by the white lines. The putative promoter region is indicated by the black box (−1926 to −1) and the minimal promoter region is indicated by the gray box (−556 to −416). The locations of the three rare variants at −803, −216, and −105 are indicated by crosses. B, DNA chromatogram and family tree of the Malay boy carrying the c.-105C>G heterozygous variant. The wild-type sequence is shown on the left (WT) and the DNA sequence of the proband is shown on the right (MUT), indicating the heterozygous variant. Squares indicate males and circles female. The shaded square indicates the proband. Yo, year old; Ht, height; Wt, weight; WFH, percent ideal weight for height. The genotypes of the proband and mother at c.-105 are indicated. The proband's fasting insulin was 16.8 mU/L (<25 mU/L), fasting glucose 3.8 mmol/L (3–6 mmol/L), 2-hour post-oral glucose tolerance test (OGTT) glucose 4.7 mmol/L (4.0–7.7 mmol/L), TC 5.07 mmol/L (<5.2 mmol/L), triglycerides (TG) 0.82 mmol/L (< 1.7 mmol/L), high-density lipoprotein-cholesterol (HDL-C) 0.82 mmol/L (1.0–1.5 mmol/L), and LDL-C 3.91 mmol/L (<2.6 mmol/L). C, DNA chromatogram and family tree of the Malay boy carrying the c.-803A>G heterozygous variant. The wild-type sequence is shown on the left (WT) and the DNA sequence of the proband is shown on the right (MUT), indicating the heterozygous variant. Squares indicate males and circles female. The shaded square indicates the proband. Yo, year old; Ht, height; Wt, weight; WFH, percent ideal weight for height. The genotypes of the proband, father, mother, and brothers at c.-803 are indicated. The proband's fasting insulin was 46.6 mU/L, fasting glucose 4.3 mmol/L, 2-hour post-OGTT glucose 4.7 mmol/L, TC 3.45 mmol/L, TG 0.75 mmol/L, HDL-C 0.93 mmol/L, and LDL-C 2.18 mmol/L. D, DNA chromatogram of the Chinese girl carrying the c.-216C>T heterozygous variant. The wild-type sequence is shown on the left (WT) and the DNA sequence of the proband is shown on the right (MUT), indicating the heterozygous variant.

nese, 36% Malay, 9% Indians, and 4% others. The mean age was 10.9 years, mean WFH 171%, and mean body mass index (BMI) 32.1 kg/m^2. Because a chart for BMI for age for local children was not available at the time of the study, WFH was used as an acceptable alternative measure of obesity (9). The research was approved by the Research and Ethics Committee of the National University Hospital, and informed consent was obtained from subjects and parents. These children had previously been screened and did not carry mutations in the coding regions of *MC3R* and *MC4R* genes (10, 11). Serum samples were obtained from study subjects in a fasted state for biochemical profiling as previously described (11).

DNA analysis

Genomic DNA was extracted from peripheral leukocytes and mutation screening was performed by direct sequencing. We screened a region 1500 bp upstream of the human *MC4R* gene as the putative promoter region, which was amplified using four overlapping amplicons of approximately 600 bp in size. Forward primers included the following: MC4PF1, 5′-CCAGCTCATGATGTTCAG-3′; MC4PF2, 5′-CAGCTACAAACTATAGAGCAC-3′, MC4PF3, 5′-CCAGCCATACCATGTCTATC-3′; and MC4PF4, 5′-GATTGGTCAGAAGGAAGCAG-3′. Reverse primers included the following: MC4PR1, 5′-GAAAGATGGCAGCAAAGTTAC-3′; MC4PR2, 5′-GAACCCAGCCAGTAGTGGTTC-3′; MC4PR3, 5′-GGCTGTAGGCGCCAGCTGCTG-3′; and MC4PR4, 5′-GGTGCAGAGAAGTGTGCATCC-3′. Amplification was carried out for 35 cycles of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 60°C, and 1 minute extension at 72°C. Sequencing was performed using a BigDye version 3.1 terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 3100 analyzer(Applied Biosystems). Novel sequence variants were confirmed by reverse sequencing.

Cloning of *MC4R* promoter reporter constructs

PCR was performed to amplify the upstream promoter region of *MC4R*. The primers used were 5′-CAT CTC GAG CCA CTA CTG GCT GGG TTC TT-3′ (forward) and 5′-GCA AGA TCT GCT GGC AGG AGA ATT CCA GT-3′ (reverse), which amplified an amplicon of 891 bp upstream from start codon ATG of the *MC4R* gene. The *MC4R* insert was then cloned into a pGL4.10 luciferase reporter vector (Promega) through *Bgl*II and *Xho*I restriction enzyme digestion. Successful clones of the wild-type *MC4R* insert and the insert with a c.-803A>G variant were obtained. The variants c.-105C>G and c.-216C>T were introduced, respectively, into the wild-type plasmid through site-directed mutagenesis (GenScript). The sequences of all plasmids were verified by DNA sequencing.

Cell transfection and luciferase assays

Human embryonic kidney 293 cells were grown to 90% confluency, and the cells were cotransfected with both *MC4R* plasmid and pGL4.74 renilla vector using lipofectamine 2000 (Invitrogen). The transfected cells were incubated at 37°C with 5% CO_2 overnight for 24 hours before they were used for the luciferase assay study. The functionality of the *MC4R* insert was determined by a dual-luciferase reporter assay (Promega) according to the manufacturer's instructions, and the assay for each *MC4R* insert was performed in triplicates.

Prediction of the functional consequences of the mutations

We investigated whether the promoter variants have any effect on putative transcription factor binding sites using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3, last accessed September 23, 2013) (12, 13).

Statistical analyses

The difference in luciferase activity between wild-type and variant *MC4R* promoter sequence was analyzed using one-way ANOVA with post hoc adjustment (Dunnett) on SPSS version 19 (SPSS).

Results

Screening of *MC4R* promoter variants

We identified two novel rare heterozygous single nucleotide substitutions, c.-105C>G (chromosome 18, 58039687) and c.-803A>G (chromosome 18, 58040385), in the *MC4R* promoter region in two unrelated Malay boys (Figure 1, B and C). Both variants were not present in 100 alleles of normal Malay individuals. The boy with the c.-105C>G variant presented a WFH of 177% at age 9 years and was overweight since the age of 4 years. He has a BMI of 27.8 kg/m^2 (WFH 136%) at age 20 years. His obese younger brother was also overweight since age 4 years, his two sisters were overweight since the age of 13 years, and his parents were overweight since their early 20s (Figure 1B). His mother is not a carrier of the c.-105C>G variant (Figure 1B).

The boy with the c.-803A>G variant presented a WFH of 225% at 9 years of age and was overweight since the age of 3 years. He had acanthosis nigricans and 74.4% body fat. His mother was severely obese (BMI 60 kg/m^2) since the age of 26 years and had type 2 diabetes mellitus; however, she is not a carrier of the c.-803A>G variant (Figure 1C). Both his father, who was moderately overweight (BMI 25 kg/m^2) since the age of 20 years, and his normal-weight younger brother are carriers of the c.-803A>G variant (Figure 1C). At age 16 years, the proband developed type 2 diabetes mellitus (glycated hemoglobin A1c [HbA1c] 11.3%) and hyperlipidemia [total cholesterol (TC) 7.21 mmol/L] and had a BMI of 61 kg/m^2 (WFH 284%). He underwent a sleeve gastrectomy at age 17 years and achieved a weight loss of 24% (165 to 125 kg) with a BMI of 45 kg/m^2 (WFH 214%) 6 months after surgery. This was associated with improvement in his metabolic profile 1 year after surgery with a HbA1c 5.8%, TC 4.50 mmol/L, and low-density lipoprotein-cholesterol (LDL-C) 2.46 mmol/L.

A rare heterozygous single-nucleotide polymorphism (SNP) rs139025325 (c.-216C>T, chromosome 18, 580396798) with a minor allele frequency (MAF) of 0.021 in Han Chinese (14)

was found in a Chinese girl with Down's syndrome (Figure 1D). Three common SNPs were also found: rs11872992 (c.-1005C>T) with a MAF of 0.356, rs8087522 (c.-896C>T) with a MAF of 0.252, and rs34114122 (c.-178A>C) with a MAF of 0.058. The proband of the c.-105C>G variant was a heterozygous carrier of the common SNP rs11872992 (c.-1005C>T). The three common SNPs were not found in the probands of the c.-803A>G and c.-216C>T variants.

The transcription factor binding site prediction program PROMO predicted the disruption of the transcription factor c-Ets-1 binding site by c.-803A>G variant because there was an increase in the dissimilarity between the variant sequence and c-Ets-1 binding site with an E score of greater than 0.05. The c.-216C>T variant was predicted to cause a disruption in p53 transcription factor binding site, whereas the c.-105C>G variant was predicted to cause a disruption in activating enhancer binding protein-2α transcription factor binding site.

Transcriptional activity of *MC4R* promoter variants

Compared with the wild type, the c.-105C>G and c.-803A>G variants showed significant reductions in transcriptional activity in vitro by 1.14- and 1.61-fold, respectively, whereas the c.-216C>T variant did not show any significant difference in transcriptional activity (Figure 2).

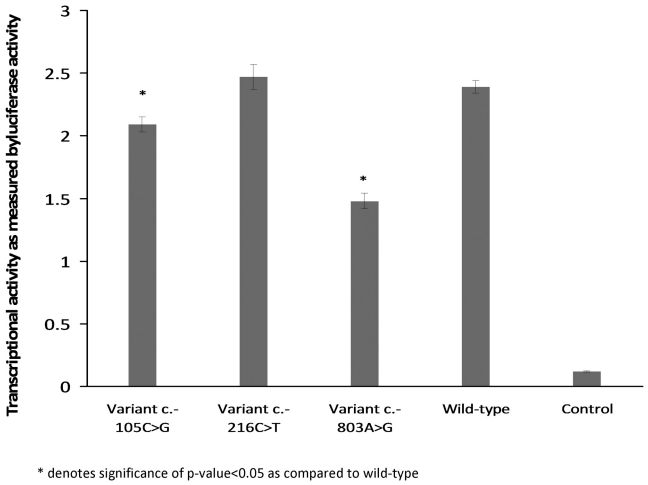

Figure 2. Basal transcriptional activities of *MC4R* promoter variants as measured in vitro by luciferase assay in transiently transfected human embryonic kidney 293 cells. The average relative transcriptional activities of the wild-type and variant *MC4R* promoters are indicated in shaded bars. The error bars indicate standard deviation (s.d.). The control represents the average relative transcriptional activity of the empty vector. The c.-105C>G, c.-216C>T, and c.-803A>G variants showed an average transcriptional activity of 2.09 ± 0.06, 2.47 ± 0.10, and 1.48 ± 0.06, respectively, whereas the wild type showed a transcriptional activity of 2.39 ± 0.05 and empty vector 0.12 ± 0.003. The asterisks indicate a significant ($P < .05$) decrease in variant *MC4R* promoter basal activity compared with wild type.

Discussion

Mutational analysis of the *MC4R* promoter region in obese human has been limited, and no mutations of functional consequence on promoter transcriptional activity has been reported so far (5). In a study of 431 obese subjects, no rare mutations were identified (6). In another study of 56 obese children, a 2-bp deletion (−439delGC) was identified in one subject (15). This −439delGC mutation was also identified in two obese individuals in a separate cohort of 152 children (16). Although the −439delGC mutation destroyed the Nhlh2 binding site, both mutant and wild-type *MC4R* promoters showed similar transcriptional activities in a reporter gene assay (16). A study of 217 Dutch obese children detected five novel variants in the *MC4R* promoter; however, no functional studies were performed (17). Interestingly, none of the five novel variants reported in the Dutch study were found in our Asian cohort.

The c.-803A>G variant did not cosegregate well with the obese phenotype in the family because the youngest sibling who carried the variant was not overweight (Figure 1C). This may be explained by incomplete penetrance, a phenomenon reported in the large cohort studies for *MC4R* mutations associated with obesity (1–3). The role of *MC4R* mutations in early-onset obesity is recognized to be influenced by gene-gene and gene-environment interactions, as illustrated by the fact that some heterozygous carriers are not obese, even for the same mutation within the same family (1–3). Perhaps the effect of the c.-803A>G variant on the father and normal-weight brother has been attenuated by other genes and/or environmental interactions. Because the proband's mother was severely obese but did not carry the c.-803A>G variant, additional genetic factors could have been inherited by the proband from his mother that worsened his obesity. For the c.-105C>G variant, its absence in the proband's overweight mother is not particularly informative. The lack of genetic testing in the other overweight members of the family is an unfortunate limitation. However, it is intriguing that the two rare variants affect transcriptional activities in vitro and lends support to their pathogenic roles in human obesity. Because the c.-216C>T variant had no effect on the transcriptional activity of the *MC4R* promoter, obesity in the proband is likely due to Down's syndrome rather than the presence of the c.-216C>T variant.

We studied a cohort of 100 individuals of the same ethnic group as controls to demonstrate the novel variants were rare (MAF < 1%). We acknowledge that a larger comparison cohort would be more informative. The SNP rs11872992 (-c.1005C>T) has been shown to have a pro-

tective effect against obesity (18), and this may attenuate the deleterious effect of the c.-105C>G variant. A limitation of our in vitro study is the lack of a construct to include both −c.105C>G and −c.1005C>T to examine the in vitro function of the novel SNP in its genetic environment.

In conclusion, we have described, for the first time, two novel human *MC4R* gene promoter variants that resulted in a decrease in basal transcriptional activity in vitro and were observed in children with early-onset obesity.

Acknowledgments

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Disclosure Summary: The authors declare no conflict of interest.

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