

ORIGINAL INVESTIGATION

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Haplosufficiency of the melanocortin-4 receptor gene in individuals with deletions of 18q

Received: 19 May 1999 / Accepted: 16 August 1999 / Published online: 7 October 1999

Abstract The melanocortin-4 receptor (MC4R) is a seven, transmembrane G-protein-coupled receptor whose ligand, α -melanocyte-stimulating hormone (α -MSH), is a post-translational derivative of pro-opiomelanocortin (POMC). The regulatory pathway, of which MC4R is a part, has become an area of intense interest because of its potential role in obesity. Three studies have identified individuals with dominantly inherited obesity segregating with mutations in the *MC4R* gene. It has been hypothesized that the mutation found in these subjects resulted in a loss of gene function resulting in obesity due to haploinsufficiency of the *MC4R* gene. We have been studying the molecular basis of the phenotype of individuals with large deletions of chromosome 18q. Due to its location at 18q21.3, the *MC4R* gene is hemizygous in approximately one-third of the individuals in our study. If hemizygosity of the *MC4R* gene results in haploinsufficiency-induced obesity, then individuals with deletions of 18q whose deletions include the *MC4R* gene should be obese in comparison with those individuals whose deletion does not include the gene. Our data indicate no difference in obesity among those deleted and not deleted for the gene. This supports the hypothesis that the MC4R gene product is haplosufficient and the involvement of MC4R in obesity may reflect a dominant negative effect.

Introduction

Obesity syndromes have been associated with mutations in the pro-opiomelanocortin (*POMC*) gene (Krude et al. 1998) and the carboxypeptidase E (*CPE*) gene, which is involved in the processing of POMC (Jackson et al. 1997). It is therefore reasonable to assume that the melanocortin-4 receptor (MC4R) might also play a role in obesity, since its ligand is derived from POMC. In addition, mice homozygous for a *Mc4r*-null allele exhibited maturity-onset obesity as well as hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar et al. 1997). The heterozygotes have an intermediate phenotype implying that the *Mc4r* gene in mice has a dose-dependent loss of function effect.

In one human study, DNA from 43 obese individuals was screened for MC4R gene mutations using single-strand conformational polymorphism (SSCP), and one individual was identified with a single mutant allele (Vaisse et al. 1998). This mutation, a heterozygous 4-bp insertion, created a truncated protein missing the sixth and seventh transmembrane domains. The same mutation was found to segregate with obesity through three generations of the propositus' family. DNA from 275 non-obese controls was screened for this mutation using a polymerase chain reaction (PCR)-based assay and none was detected.

In an additional study, DNA from 63 obese individuals was screened for mutations in the *MC4R* gene using direct nucleotide sequencing (Yeo et al. 1998). One individual was identified who was heterozygous for a 4-bp deletion in the region encoding the fifth transmembrane domain of the *MC4R* gene. The propositus' father, who had been obese since childhood, was found to have the identical frame-shift mutation. The authors hypothesized that since the truncated protein is a receptor, thought to act as a monomer, it was likely to have a haploinsufficient mechanism of disease. Combined with the evidence of haploinsufficiency in the mouse, this appeared to be a reasonable hypothesis.

In a more recent study, the DNA from 306 extremely obese children and adolescents was screened by means of

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SSCP for mutations in the *MC4R* gene (Hinney et al. 1999). Nine individuals from eight families were found to have a variety of *MC4R* mutations. Five of the eight mutations were thought to give rise to a truncated protein, prompting the authors to speculate that the mechanism of action of these mutations was haploinsufficiency. The other three mutations were missense mutations, whose mechanism of causing the obese phenotype remained to be determined.

A remarkable aspect of these three studies is that the phenotype resulting from *MC4R* mutations is not a tendency toward increased body mass, but is extreme obesity. The effect of mutations in this gene are profound and not merely a skewing from average. However, none of these studies has provided proof of haploinsufficiency. Definitive proof would be obesity in the absence of any gene product from the mutant allele; that is, subjects hemizygous for the *MC4R* gene with extreme obesity would provide proof of haploinsufficiency.

Subjects, materials and methods

Subjects

Individuals with deletions of 18 were recruited through the Chromosome 18 Registry and Research Society or through their private physician. The study was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio and the Audie L. Murphy Veterans Administration Hospital. Written informed consent was obtained from all subjects.

Molecular analysis

The molecular analysis performed to determine the extent of the deletion has been previously described (Cody et al. 1997). The *MC4R* BAC clone used to perform the fluorescence in situ hybridization (FISH) analysis was identified by screening the bacterial artificial chromosome (BAC) library (Research Genetics) with primers designed from the 3' untranslated region of the cDNA sequence (*MC4R*-A: 5'GACAGAGCACGCAATATAGGA3'; and *MC4R*-B: 5'GGAATACTCAACCAGTACCCT3').

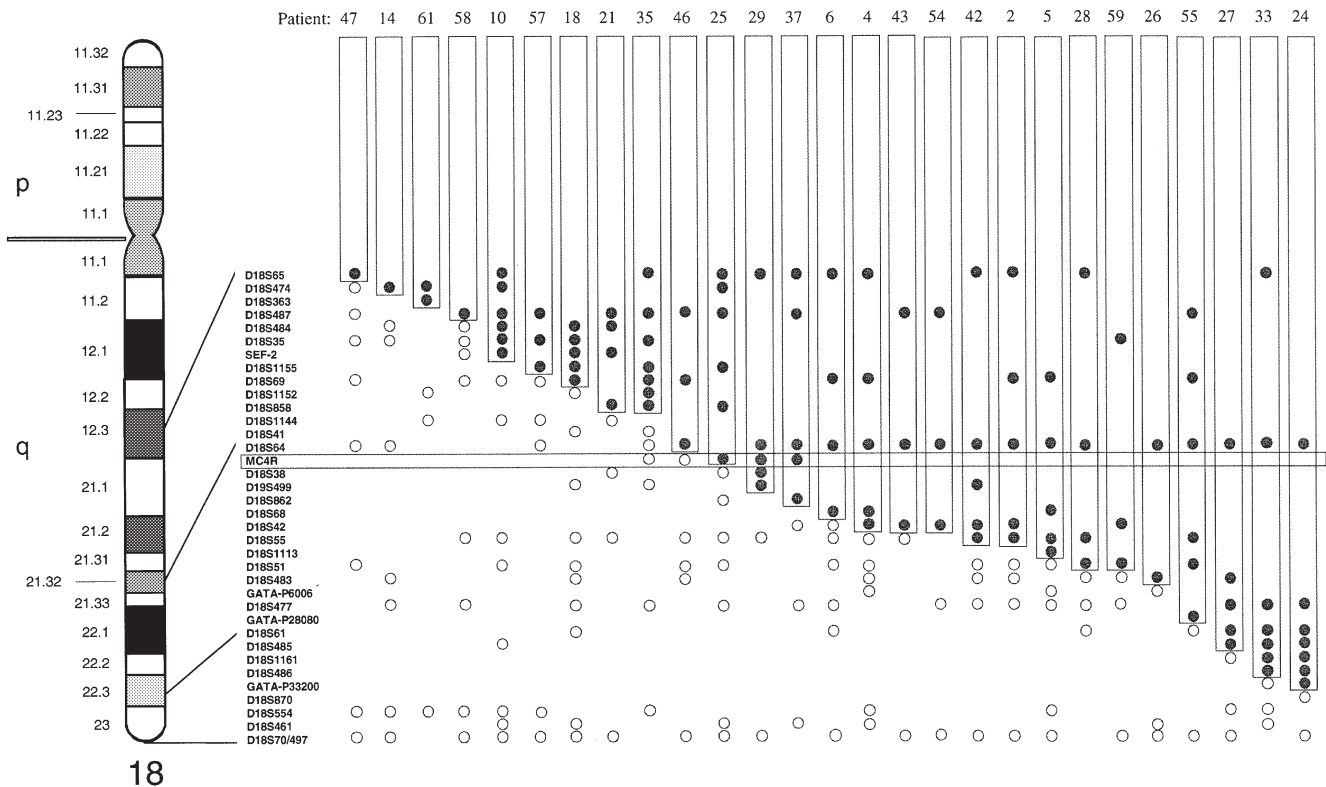
Fluorescence in situ hybridization

The chromosomes from subjects whose breakpoints were near *MC4R* were studied by means of FISH using a BAC genomic clone containing the *MC4R* gene. These methods have been described previously (Gay et al. 1997).

Statistics

Z scores for height and weight were determined using the normative data presented in "Handbook of Normal Physical Measurements" (Hall et al. 1989). Standard deviations (SD) were estimated as one quarter of the distance between the 95th and 5th or 97th and

Fig. 1 Molecular characterization of the extent of the 18q deletion using polymerase chain reaction (PCR)-based polymorphic markers and fluorescence in situ hybridization (FISH). Each rectangle represents the minimum estimate of the size of the chromosome for the patient whose number is at the top of the rectangle. Open circles indicate the presence of one allele and black circles indicate the presence of two alleles. The data from uninformative markers are not shown. The melanocortin-4 receptor (*MC4R*) genotype was gathered using a FISH probe; the remainder of the data is PCR-based marker analysis



3rd percentiles for age, depending on the available normative data. The height-age Z scores were determined as described above, except, instead of using the subject's chronological age, the age at which the subject's height is at the 50% percentile was used for comparison with norms.

Results

To determine whether hemizygosity for the *MC4R* gene causes obesity, we compared the height and weight of 27 individuals over the age of 1 year, who had terminal deletions of chromosome 18q. Ten individuals had deletions that included the *MC4R* gene and 17 individuals had deletions that did not include the gene. Figure 1 is a diagrammatic representation of the extent of each individual's deletion. Except for the *MC4R* genotype, these data were gathered using polymorphic PCR-based markers. The *MC4R* data in Fig. 1 was gathered using FISH with a BAC probe containing the *MC4R* gene.

Figure 2 shows participant growth data in the same order as in Fig. 1. If hemizygosity for the *MC4R* gene causes obesity resulting from haploinsufficiency, then the individuals hemizygous for *MC4R* (shown in *black*) should be both taller and heavier than those not missing the gene (shown in *white*). When the two groups were compared using age-related standard deviations (Z scores) for height

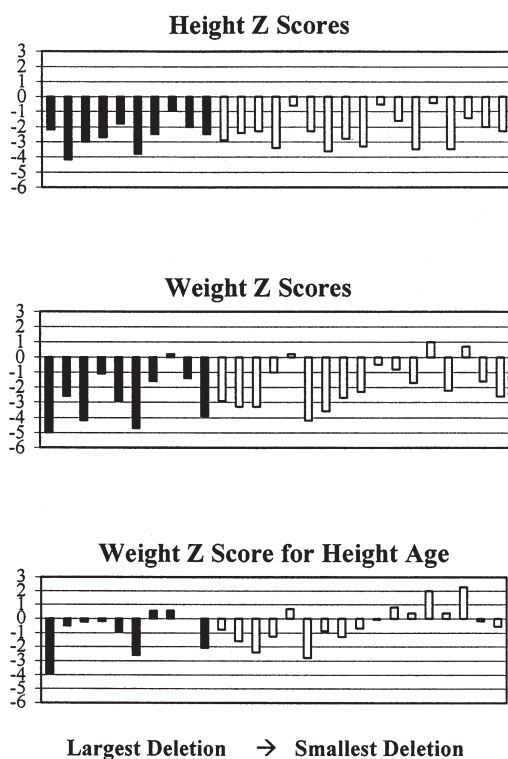


Fig. 2 Growth data for individuals with terminal deletions of 18q. The patient data are arranged in the same *left to right* order as in Fig. 1, with the data from the child with the largest deletion on the *left* and the child with the smallest deletion on the *right*. Individuals with deletions that include the melanocortin-4 receptor (*MC4R*) gene are shown in *black* and those whose deletion is too small to include *MC4R* are shown in *white*

and weight, there was no significant difference between the two groups using a one-tailed *t*-test. The *P* value for the height comparison between the two groups was 0.25. The *P* value for the weight comparison between the two groups was 0.08. Because children are short, we also compared their weight Z scores after normalizing for their height. Again, there was no difference between those individuals whose deletion did and did not include the *MC4R* gene (*P*=0.15). Based on these data, it appears that *MC4R* is haplosufficient in patients with deletions of 18q.

Discussion

Yeo et al. (1998), Vaisse et al. (1998), and Hinney et al. (1999) discovered mutations in the *MC4R* gene in several extremely obese individuals. Since many of these mutations were expected to result in a truncated protein, Yeo et al. (1998) and Hinney et al. (1999) hypothesized that the obesity was due to haploinsufficiency. Diseases resulting from truncated protein mutations do not necessarily have a loss of function mechanism. A truncated protein could have either a loss or gain of function. Loss of function can only be implied when patients found to have whole gene deletions have identical phenotypes to those with truncated protein. If patients with whole gene deletions do not have the same phenotype, then a gain of function for a truncated protein is inferred. It is possible that patients with whole gene deletions might not have an abnormal phenotype. This demonstrates haplosufficiency, which occurs in carriers of recessive mutations and is therefore a common occurrence.

In this study, we were testing the hypothesis that haploinsufficiency of the *MC4R* causes obesity. We studied a group of individuals with chromosome 18q deletions, some of whom have deletions that include the *MC4R* gene and some whose deletions do not include the gene. Our comparison of these two groups demonstrated no significant difference in weight between those whose deletion included *MC4R* and those that do not. Thus, no haploinsufficiency was evident in our population.

It could be argued that other genes on 18q might override the ability of the *MC4R* gene to affect growth and thereby mask differences between the two groups. Such genes could be in the *MC4R* growth pathway or involved in other pathways with a stronger effect on body mass. If such a gene does exist, it would be located distal to *MC4R* and would be an important gene to identify. However, identification of such a gene is beyond the scope of this study.

Alternatively, the obese individuals may have mutations of other genes that the individuals with 18q deletions do not have, that moderate the effect of the *MC4R* mutation. If this were the case, the obesity would be polygenic and not the result of simple haploinsufficiency.

It is also possible that we missed a true effect of *MC4R* due to small sample size. The given sample would have 80% power to detect a relatively large difference in means, where the distribution of measurements in the two groups

(group 1 = deleted *MC4R* and group 2 = non-deleted *MC4R*) overlapped by no more than 50% (Cohen 1988). This effect translates into a standardized difference of approximately 1.0 ([mean of group 1 - mean of group 2] / standard deviation). If haploinsufficiency of *MC4R* indeed caused obesity, this large effect would be likely. In our data, we actually see a trend in the opposite direction, where subjects with two copies of *MC4R* were somewhat taller and heavier than those with one copy of *MC4R*.

The possibility exists that the remaining allele in our participants is somehow upregulated and therefore haplo-sufficient. If this were true, dosage compensation of the *MC4R* gene would also be possible in the individuals cited by Yeo et al. (1998), Vaisse et al. (1998), and Hinney et al. (1999). This theory cannot be tested experimentally, since the only available tissues for gene expression studies are lymphoblast cell lines, which do not express *MC4R*. However, dosage compensation has rarely been observed in monosomy syndromes (Wang et al. 1999).

Understanding the types of mutations and the mechanism by which those mutations cause an abnormal phenotype is important for understanding the pathophysiology of G-protein-coupled receptor-related disease. The data presented here adds to the growing body of knowledge about the *MC4R* gene and its mode of action. The mutations in *MC4R* detected by Yeo et al. (1998), Vaisse et al. (1998), and Hinney et al. (1999) may indeed be loss of function mutations, but our data would suggest that these mutations must have a dominant-negative effect as opposed to a null-allele effect. Their data does not rule out the possibility of a dominant-negative mechanism. To obtain definitive proof of haploinsufficiency, one must identify an individual with the phenotype who has a mutation prohibiting formation of a gene product or a deletion of the gene. While other G-protein-coupled receptors have been shown to have loss of function mutations, none has definitively been shown to cause disease by haploinsufficiency (Spiegel 1996).

Acknowledgements We would like to thank the MacDonald family and Microsoft Corp. for their generous support of this work. Participants were assessed at the General Clinical Research Center, Audie Murphy Veteran Administration Hospital, which is supported by NIH grant 2M01RR01346-18.

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