Mutational Analysis of the Melanocortin-4 Receptor Gene in Chinese Children with Obesity

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Objectives. The melanocortin system is involved in physiological regulation of homeostasis. The melanocortin-4 receptor (MC4R) in the hypothalamus is thought to be important in controlling feeding behavior. Genetic studies have identified two mutations, Ser-30-Phe and Asp-298-Asn, of MC4-R which are associated with dominant forms of inherited obesity.

Methods. In order to examine whether these two mutations are associated with the development of early-onset obesity in the Chinese population, we used a polymerase chain reaction-base analysis to analyze the MC4R genotypes of 92 obese and 185 non-obese children.

Results. No mutations were detected in any of the individuals that were screened in this study. However, two novel polymorphisms, Ile-103 and Ile-297, were identified in both obese and normal individuals which were associated with the phenotypes under study.

Conclusions. It seems that the Ser-30-Phe and Asp-298-Asn mutations do not contribute to inherited susceptibility to early-onset obesity in Chinese children. **(Mid Taiwan J Med 2003;8:66-72)**

Key words

Chinese, early onset obesity, MC4-R, polymerase chain reaction

INTRODUCTION

Obesity is a serious health problem that causes complications such as type 2 diabetes, hypertension, atherosclerosis and neoplasms [1]. Reports indicate that the prevalence of obesity in childhood has been increasing in recent years in Taiwan [2] and Western countries [3,4]. Also, there is evidence that obesity originates in childhood and tracks to adulthood [5,6].

The most common forms of human obesity arise from the interaction of multiple genes, environmental factors, and behavior [7]. Body weight regulation is a complex biological system in which food intake and energy expenditure depend on the interaction of many independent regulatory pathways. Data from studies on families with twins have indicated that the development of obesity is significantly influenced by genetic factors [8,9]. About 40% to 70% of the variations in obesity-related phenotypes, such as body mass index, sum of skinfold thickness and fat mass are heritable [10,11]. Genes that have been implicated in the control of fat-mass production include hypothalamic neurotrans-

Received : February 21, 2003. Revised : March 21, 2003.
Accepted : March 22, 2003.

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mitters and steroid hormone receptors [12,13]. Evidence accumulated from murine and human genetic models suggest that melanocortins and melanocortin receptors (MCR) play an important role in regulating body weight [14-17]. Of the five known melanocortin receptors, the melanocortin-4 receptor (MC4-R) has been most closely linked to body weight regulation.

The MC4-R is a 332-amino acid protein encoded by a single exon gene localized on chromosome 18q22 [18,19]. It is a G protein-coupled, seven-transmembrane receptor expressed in a number of hypothalamic sites [20,21], and plays an important role in regulating feeding behavior [22-24]. There are four other MCR subtypes that have been identified: the MC1-R [25], MC2-R [26], MC3-R [27,28], and MC5-R [29,30], which differ in their tissue distribution as well as physiological function. MC4-R integrates an agonist signal provided by an alpha-melanocyte-stimulating hormone (α-MSH) [24], a neuropeptide derived from POMC [31]. Inactivation of this receptor by gene targeting results in obesity in mice [23]. However, the association of the MC4-R gene with human obesity is still controversial [32-34].

Mutations of POMC were found to be associated with a human syndrome that is characterized by severe early-onset obesity [35]. It is therefore reasonable to assume that MC4-R also plays a role in early-onset obesity. Recently, two missense mutations, Ser-30-Phe and Asp-298-Asn in the MC4-R, were detected and were assumed to lead to the phenotype of extreme obesity [14,36]. Ser-30-Phe is located at the extracelluar domain of the receptor, where it harbors 3 N-linked glycosylation sites. Asp-298-Asn was identified in the 7-transmembrane domain, a region highly conserved among MCR genes [14], and was found to be associated with fatter, higher-feed consuming and faster-growing animals. To examine whether these two missense variants in the MC4-R gene are associated with early-onset obesity, we analyzed the MC4-R genotypes of elementary school children in Taiwan using a polymerase chain reaction-based assay.

SUBJECTS AND METHODS

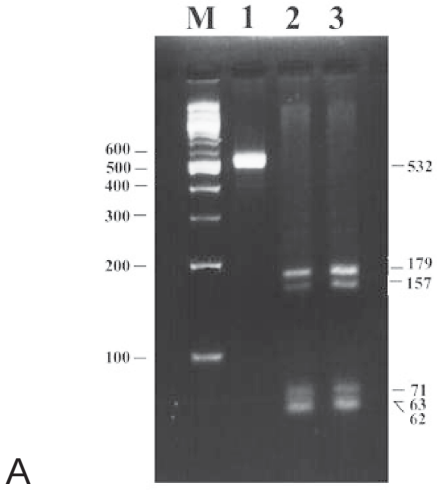
Study Design and Population

A cross-sectional study was conducted from March to April 2000 in Taichung city, Taiwan. The population of this study consisted of first graders from elementary schools in northern Taichung city. A total of 2,230 students were recruited. All selected students were given a structured questionnaire to take home to their parents with a cover letter briefly describing the study. Those parents who approved of their children participating in this study would sign their consent forms before they filled out the questionnaires. Information collected by the questionnaires consisted of demographic factors and medical history. The clinical examination included measurements of sitting blood pressure (with a random-zero sphygmomanometer), height, and weight.

Body mass index (BMI) was calculated as follows: BMI = weight (kg)/height $(m)^2$. Obesity was defined as a BMI greater than or equal to the 95th percentile [37]. Ninety-two obese and 185 non-obese children (BMI between 45th and 55th percentile) were selected for this study.

DNA Isolation and Analysis of the MC4R Mutation

DNA was extracted using a standard method as previously described [38]. A 532-bp fragment containing the Ser-30-Phe mutation was amplified by PCR, using the forward primer 5'-GATGCACACTTCTCTGCACC-3' and the reverse primer 5'-GATGAACAAAATGCCTGAAACC-3'. A 539-bp fragment containing the Asp-298-Asn mutation was amplified by PCR, using the forward primer 5'-CTTCTATGCTCTCCAGTACC-3' and the reverse primer 5'-GCTAGACAAGTCACAAAGG-3'. The amplification conditions were: 35 cycles of denaturation at 94°C for 2 min, annealing at 52°C for 2 min, and extension at 72°C for 3 min with a final extension at 72°C for 7 min. In order to differentiate between normal and mutated alleles, the 532-bp PCR products were digested with *Hinf*I restriction enzyme and the 539-bp PCR products were digested with *Cla*I restriction enzyme. The digested PCR products were then analyzed on a



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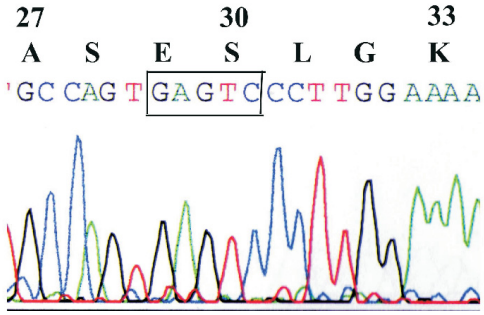

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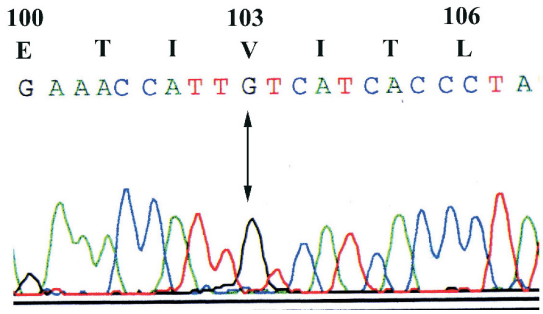

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Fig. 1. A: PCR-based analysis of Ser-30-Phe genetic alteration. The N-terminus region of the MC4-R was amplified by PCR which resulted in a 532-bp fragment (lane 1). After digesting with *Hinf*I, the wild type was cut into five fragments, 62-bp, 63-bp, 71-bp, 157-bp and 179-bp (lanes 2 & 3). Lanes 2 and 4, samples from an obese and a normal child, respectively. M, marker, 100-bp ladder; B: Direct sequencing analysis of the possible mutagenic region. The upper numbers indicate the corresponding amino acid sequence. The boxed area is the *Hinf*I cutting site; C: A→G substitution, marked by the arrow heads, was found in codon 103.

2% agarose gel and visualized with ethidium bromide.

Sequencing Analysis

The PCR products were extracted using a QIA quick PCR purification kit (QIAEN, Inc., Valencia, CA) as described by the manufacture. The purified products were sequenced using the di-deoxy chain termination method as described in the BigDye™ Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) and the samples were analyzed by an ABI PRISM™ 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA).

RESULTS

We analyzed DNA from 92 obese and 185 non-obese children based on their BMI. The PCR reactions amplified the potential mutation regions: a 532-bp fragment (Fig. 1A, lane 1) containing codon 30 and a 539-bp fragment (Fig. 2A, lane 1) containing codon 298. To detect the C→T substitution in codon 30, the 532-bp fragment was digested with *Hinf*I restriction enzyme. The normal allele was cleaved into five fragments, 62-bp, 63-bp, 71-bp, 157-bp and 179-bp (Fig. 1, lanes 2 & 3), whereas the mutant allele gave only four fragments, 63-bp, 133-bp, 157-bp and 179-bp. To analyze the codon 298 G→A substitution, we digested the 539-bp PCR product with *Cla*I restriction enzyme. If the normal allele contained a *Cla*I site, the PCR fragment would be cleaved, giving two fragments of 99- and 438-bp (Fig. 2, lane 2). On the other hand, if the mutant allele were not cut, the fragment would remain 539-bp.

None of the PCR products from the 277 children that we screened by this method contained the C→T substitution in codon 30. The PCR products were then sequenced and the results showed that all these PCR products were normal (Fig. 1B). While screening possible G→A substitutions in codon 298, we found only one individual whose BMI was in normal range who carried the heterozyous genotype (Fig. 2, lane 3). However, data from direct sequencing of the PCR product revealed a T→C alteration in codon 297

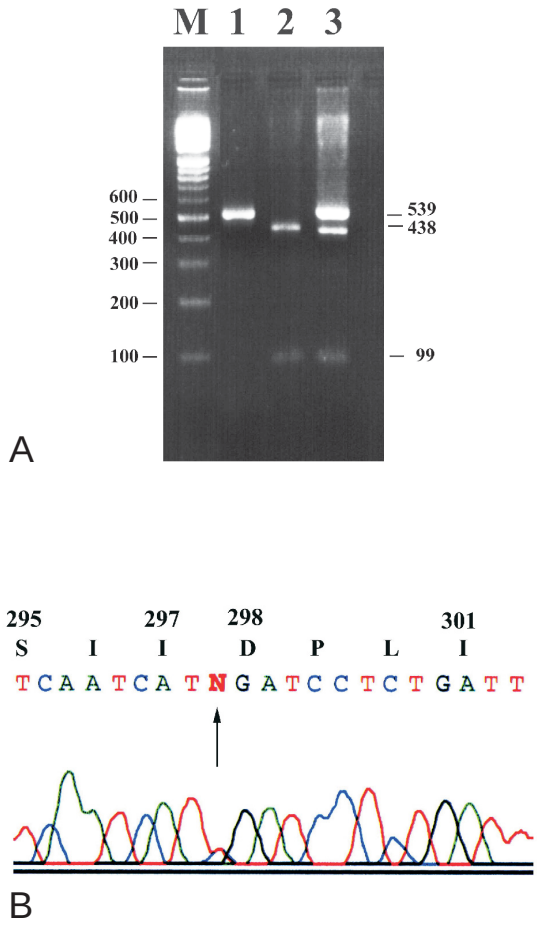

Fig. 2. A: PCR-based analysis of Asp-298-Asn genetic alteration. The N-terminus region of the MC4-R was amplified by PCR which resulted in a 539-bp fragment (lane 1). After digesting with *Cla*I, the wild type was cut into two fragments, 99- and 438-bp (lane 3). Lane 2 is the result from a heterozygous individual. M, marker, 100-bp ladder; B: Direct sequencing analysis of the possible mutagenic region. The upper numbers indicate the corresponding amino acid sequence. The arrow head points to the polymorphic site.

instead of a G→A substitution in codon 298 (Fig. 2C).

A new polymorphic region was detected by sequencing the 532-bp PCR fragment. All specimens contained an A→G substitution in codon 103 which resulted in an amino acid change from isoleucine (ATC) to valine (GTC). We analyzed 13 unrelated individuals of Australian, Philippine, Thai, and Caucasian origins and all of them carried the Val/Val genotype.

DISCUSSION

Previous studies [36,14] reported that individuals who carry C→T mutations in codon 30 (Ser-30-Phe) and G→A mutations in codon 298 (Asp-298-Asn) in the MC4-R gene may be associated with dominantly inherited obesity. In this study, we investigated this hypothesis in the Chinese population using a PCR-based assay. We found no genetic alteration in codon 30 or codon 298. It seems that these two mutations do not contribute to inherited susceptibility to early-onset obesity.

The fact that we detected no genetic abnormalities in extremely obese young children can be explained in two ways. First, since the frequencies of these two mutations are low, they may not be related to inherited obesity. Second, these two mutations may vary according to ethnic groups. The population in our study was predominantly Chinese and it is possible that the frequency of mutation is only higher in Caucasians.

Two novel polymorphic sites were identified in this study. We found a T→C substitution in codon 297 but this alteration did not result in any amino acid change. The analysis of the Ser-30-Phe mutation led to the identification of a new polymorphic site A→G in codon 103. Even though screening of four other different ethnic groups also yielded the same genotype, it is possible that this alteration was due to ethnic differences. Larger epidemiological studies should be undertaken among a variety of populations.

ACKNOWLEDGMENTS

This work was supported by grants from the China Medical College Hospital (DMR89-052 & DMR90-099).

REFERENCES

1. Dietz WH. Health consequences of obesity in youth: childhood predictors of adult disease. [Review] *Pediatrics* 1998;101:518-25.
2. Chen W. Childhood obesity in Taiwan. *Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi* 1997;38:438-42.

3. Troiano RP, Flegal KM. Overweight children and adolescents: description, epidemiology, and demographics. [Review] *Pediatrics* 1998;101:497-504.
4. Reilly JJ, Dorosty AR. Epidemic of obesity in UK children. *Lancet* 1999;354:1874-5.
5. Clarke WR, Lauer RM. Does childhood obesity track into adulthood? *Crit Rev Food Sci Nutr* 1993;33:423-30.
6. Serdula MK, Ivery D, Coates RJ, et al. Do obese children become obese adults? A review of the literature. [Review] *Prev Med* 1993;22:167-77.
7. Price RA. Regulation of body weight: Biological and behavioral mechanism. In: Bouchard C, Bray GA, eds. Wiley: Chichester, UK.
8. Moll PP, Burns TL, Lauer RM. The genetic and environmental sources of body mass index variability: the Muscatine Ponderosity Family Study. *Am J Hum Genet* 1991;49:1243-55.
9. Stunkard AJ, Harris JR, Pedersen NL, et al. The body-mass index of twins who have been reared apart. *N Engl J Med* 1990;322:1483-7.
10. Allison DB, Kaprio J, Korkeila M, et al. The heritability of body mass index among an international sample of monozygotic twins reared apart. *Int J Obes Relat Metab Disord* 1996;20:501-6.
11. Comuzzie AG, Blangero J, Mahaney MC, et al. Genetic and environmental correlations among hormone levels and measures of body fat accumulation and topography. *J Clin Endocrinol Metab* 1996;81:597-600.
12. Tritos NA, Maratos-Flier E. Two important systems in energy homeostasis: melanocortins and melanin-concentrating hormone. [Review] *Neuropeptides* 1999;33:339-49.
13. Wilson BD, Bagnol D, Kaelin CB, et al. Physiological and anatomical circuitry between Agouti-related protein and leptin signaling. *Endocrinology* 1999;140:2387-97.
14. Kim KS, Larsen N, Short T, et al. A missense variant of the porcine melanocortin-4 receptor (MC4R) gene is associated with fatness, growth, and feed intake traits. *Mamm Genome* 2000;11:131-5.
15. Ollmann MM, Wilson BD, Yang YK, et al. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 1997;278:135-8.
16. Ollmann MM, Lamoreux ML, Wilson BD, et al. Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. *Genes Dev* 1998;12:316-30.
17. Ho G, MacKenzie RG. Functional characterization of mutations in melanocortin-4 receptor associated with human obesity. *J Biol Chem* 1999;274:35816-22.
18. Gantz I, Miwa H, Konda Y, et al. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J Biol Chem* 1993;268:15174-9.
19. Sundaramurthy D, Campbell DA, Leek JP, et al. Assignment of the melanocortin 4 receptor (MC4R) gene to human chromosome band 18q22 by in situ hybridisation and radiation hybrid mapping. *Cytogenet Cell Genet* 1998;82:97-8.
20. Tatro JB. Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. [Review] *Neuroimmunomodulation* 1996;3:259-84.
21. Mountjoy KG, Mortrud MT, Low MJ, et al. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol* 1994;8:1298-308.
22. Bray GA. Hypothalamic and genetic obesity: an appraisal of the autonomic hypothesis and the endocrine hypothesis. [Review] *Int J Obes* 1984; 8(Suppl 1):119-37.
23. Huszar D, Lynch CA, Fairchild-Huntress V, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 1997;88:131-41.
24. Fan W, Boston BA, Kesterson RA, et al. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997;385:165-8.
25. Lu D, Willard D, Patel IR, et al. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* 1994;371:799-802.
26. Mountjoy KG, Robbins LS, Mortrud MT, et al. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992;257:1248-51.
27. Gantz I, Konda Y, Tashiro T, et al. Molecular cloning of a novel melanocortin receptor. *J Biol Chem* 1993; 268:8246-50.
28. Roselli-Rehfuss L, Mountjoy KG, Robbins LS, et al. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci USA* 1993;90:8856-60.
29. Gantz I, Shimoto Y, Konda Y, et al. Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochem Biophys Res Commun* 1994;200:1214-20.
30. Chhajlani V, Muceniece R, Wikberg JE. Molecular cloning of a novel human melanocortin receptor.

Biochem Biophys Res Commun 1993;195:866-73.
31. Keightley MC, Funder JW, Fuller PJ. Molecular cloning and sequencing of a guinea-pig pro-opiomelanocortin cDNA. *Mol Cell Endocrinol* 1991; 82:89-98.
32. Gu W, Tu Z, Kleyn PW, et al. Identification and functional analysis of novel human melanocortin-4 receptor variants. *Diabetes* 1999;48:635-9.
33. Sina M, Hinney A, Ziegler A, et al. Phenotypes in three pedigrees with autosomal dominant obesity caused by haploinsufficiency mutations in the melanocortin-4 receptor gene. *Am J Hum Genet* 1999;65:1501-7.
34. Norman RA, Permana P, Tanizawa Y, et al. Absence of genetic variation in some obesity candidate genes (GLP1R, ASIP, MC4R, MC5R) among Pima indians. *Int J Obes Relat Metab Disord* 1999;23:163-5.
35. Krude H, Biebermann H, Luck W, et al. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* 1998;19:155-7.
36. Hinney A, Schmidt A, Nottebom K, et al. Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. *J Clin Endocrinol Metab* 1999;84:1483-6.
37. Barlow SE, Dietz WH. Obesity evaluation and treatment: Expert Committee recommendations. The Maternal and Child Health Bureau, Health Resources and Services Administration and the Department of Health and Human Services. *Pediatrics* 1998;102:E29.
38. Wang NM, Yeh KT, Tsai CH, et al. No evidence of correlation between mutation at codon 531 of src and the risk of colon cancer in Chinese. *Cancer Lett* 2000; 150:201-4.

台灣肥胖學童Melanocortin-4 Receptor基因多形性分析

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目的 Melanocortin系統與身體熱量平衡的調節有關，在下視丘的Melanocortin-4受體(MC4-R)被認爲與攝食行爲有密切關係。最近的基因研究顯示Ser-30-Phe及Asp-298-Asn這兩個突變與顯性遺傳型的肥胖有關。

方法 爲檢驗上述兩種突變是否爲中國人早發性肥胖之因子，我們以聚合酶鏈鎖反應基分析法分析92位肥胖與185位非肥胖小孩之MC4R基因型。

結果 所有受檢小孩並未發現有上述的突變現象。進一步分析在肥胖者及非肥胖者均發現兩個新的突變：Ile-103及Ile-297。

結論 Ser-30-Phe及Asp-298-Asn的突變似與中國人種的體重調節無關。（中台灣醫誌 **2003;8:66-72**）

關鍵詞

中國人，早發性肥胖，MC4-R，聚合酶連鎖反應

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收文日期：2003年2月21日 修改日期：2003年3月21日

接受日期：2003年3月22日