Molecular screening of both the promoter and the protein coding regions in the human ob gene in Japanese obese subjects with non-insulin-dependent diabetes mellitus

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Abstract

Objective: Although the molecular mechanism of obesity has been poorly understood, recent studies indicate that leptin plays a critical role in regulating both food intake and body weight. Because obesity decreases the sensitivity to insulin, the human ob gene is presumed to be one of the candidate genes for non-insulin-dependent diabetes mellitus (NIDDM) associated with obesity. Although the protein coding region in the ob gene has been screened for mutations, the promoter region and the non-coding first exon have not yet been studied. We investigated the involvement of the human ob gene, especially mutations at the promoter region and the non-coding first exon, in the development of NIDDM associated with obesity.

Subjects: The study group comprised 60 Japanese obese subjects with NIDDM (body mass index (BMI) 43.6 ± 26.4, 29.0 ± 0.41 (mean ± S.E.M.)) and 24 obese individuals with impaired glucose tolerance (IGT) (30 ± 26.4, 27.1 ± 0.22).

Methods: Mutations at both the promoter region and all three exons in the human ob gene were screened by the single-stranded conformational polymorphism analysis. When aberrantly migrated bands were recognized, the PCR-amplified DNA fragment was directly sequenced.

Results: In the protein coding region a silent mutation in the second exon was detected. The non-coding first exon and the about 100 bp 5'-flanking region of the gene which contains a proximal CCAAT/enhancer-binding protein site were screened, but no mutations were found.

Conclusion: These results suggest that no mutations in either the promoter region at the about 100 bp 5'-flanking region of the gene, or in any of the three exons, are involved in the development of NIDDM or IGT associated with obesity.

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Introduction

Obesity is a critical public health problem. While familial aggregation and twin studies (1, 2) have suggested the involvement of a genetic factor in the etiology of obesity, recent cloning of the genes responsible for obesity greatly facilitated molecular investigations targeted to the inherited component of human obesity. The ob gene is expressed exclusively in adipose tissue, the product of which is a secretory protein of 167 amino acids named leptin. Obesity in the ob/ob mouse has been demonstrated to result from a nonsense mutation in the ob gene or total absence of ob mRNA (3). The ob gene is overexpressed and plasma leptin levels increased in obese humans and genetically obese rodents (4, 5). Recombinant leptin administered to ob/ob mice decreases both food intake and body weight, whereas injected leptin does not influence food intake in db/db mice (6, 7), in which a mutation in the leptin receptor gene was identified (8). Thus, this protein could be thought to function via its specific receptor in the hypothalamic region to control appetite and energy expenditure, thereby regulating body weight.

It is well known that obesity induces and/or exacerbates diabetes mellitus through induction of insulin resistance. Thus, it is reasonable to assume that the ob gene is one of the candidate genes for susceptibility to diabetes mellitus associated with obesity. To test this possibility, we screened Japanese non-insulin-dependent diabetes mellitus (NIDDM)
subjects with obesity for mutations in the human ob gene, especially at the promoter region.

**Subjects and methods**

The study comprised 60 Japanese subjects with NIDDM and 24 individuals with impaired glucose tolerance (IGT), both groups of which exhibited obesity (body mass index (BMI) 43.6 ± 26.4, 29.0 ± 0.41 (mean ± S.E.M.) and 30 ± BMI ≥ 26.4, 27.1 ± 0.22, respectively and those including 23 subjects with BMI ≥ 30) at the time of study. Diabetes mellitus was diagnosed according to criteria of the World Health Organization Expert Committee on Diabetes Mellitus (9). Informed consent was obtained from the study subjects. Genomic DNA was extracted from peripheral blood cells. Each exon and 5′-untranslated region of the ob gene was PCR-amplified using [γ-32P]ATP-endolabeled primers specific for the ob gene (Table 1) and subjected to single-stranded conformational polymorphism (SSCP) as previously described (10).

PCR reaction included 100 ng genomic DNA template, 0.2 μmol/l of each primer, which had been labeled with [γ-32P]ATP, 0.2 mmol/l of each nucleotide triphosphate, 0.06 unit of Taq DNA polymerase and reaction buffer supplied by the manufacturer (Perkin Elmer Cetus, Norwalk, CT, USA) in a total volume of 5 μl. The PCR conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 74 °C for 2 min, with a final extension for 7 min.

The PCR products were diluted 10-fold with formamide buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 95 °C for 3 min, and 1.5 μl of each sample was loaded onto a 5% non-denaturing polyacrylamide gel. Each sample was electrophoresed under combined four conditions (room temperature or 4 °C; with or without 10% glycerol). The gels were dried and exposed to films (XAR-5; Kodak) with an intensifying screen for 12 h at −70 °C. When aberrantly migrated bands were found, the amplified PCR products were directly sequenced by a DNA sequencer (Applied Biosystems, Foster City, CA, USA).

### Table 1 Oligonucleotide primers used for PCR-SSCP to screen mutations in the human ob gene.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide sequence</th>
<th>Size of product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sense 5′-GGAGGCTGGCGCTAGAAATG-3′ Antisense 5′-GGCCCTACCTTGCAACCGT-3′</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>Sense 5′-TCTGTTCAGGGCCCAAGA-3′ Antisense 5′-GCCGGGGAATCTGCTCCCTA-3′</td>
<td>203</td>
</tr>
<tr>
<td>3</td>
<td>Sense 5′-TCCTCTTCCCTCTGCA-3′ Antisense 5′-GCAGGAAGAGTGACCTTCA-3′</td>
<td>524</td>
</tr>
</tbody>
</table>

**Results and discussion**

Using the SSCP method, no aberrantly migrated bands were recognized at the about 100 bp 5′-flanking region of the first exon. Furthermore, no mutations were recognized in the first and third exons, while a silent mutation (CA(A(Gln)→CAG(Gln)) in codon 25 in the second exon was observed in one case with IGT, which is identical to that reported by Maffei et al. (11).

In the present study, we could not find any significant mutation at the protein coding region in the human ob gene in Japanese NIDDM patients with obesity. Stirling et al. (12) have identified microsatellite markers near the human ob gene and these markers were typed in Mexican–American NIDDM-affected sib pairs. Using these polymorphic markers, no evidence of linkage or association was present between either marker and NIDDM. Furthermore, recent reports using the SSCP method showed that mutations in exons 2 and 3 of the ob gene were absent in Japanese and Asian Indian NIDDM patients associated with obesity (13). These data suggest that mutations in the protein coding region in the ob gene are not likely to be a cause of obesity or NIDDM, which is consistent with the results in the present study.

In spite of these reports elucidating the protein coding region in the human ob gene, the involvement of the promoter region in the development of NIDDM or obesity has not yet been studied. Although there are several cis-acting regulatory DNA elements such as Sp-1-like sequences, CCAAT/enhancer-binding protein sites (C/EBP), glucocorticoid response element (GRE), and cAMP response element-binding protein sites (CREB) at a 3 kb 5′-flanking region of the gene (14), as little as 217 bp of DNA upstream of the transcription start site are sufficient to control basal and tissue-specific ob gene expression (15). Especially, a C/EBP site most proximal to the transcription start site was shown to be functional and important for high level expression of the ob gene in pre-adipocytes and adipocytes. Because the promoter region screened by the SSCP method in the present study contains this proximal C/EBP site, mutations of this cis-acting DNA element are unlikely to be a cause of the development of NIDDM or obesity. In addition, the present findings suggest that other
regulatory elements near this C/EBP site are not involved in a cause of NIDDM or obesity. It does not necessarily mean, however, that mutations in the ob gene are not a cause of NIDDM or obesity at all, but it cannot be ruled out that mutations of this gene might be a cause of obesity in a limited number of pedigrees. Furthermore, it is also possible that mutations of several other regulatory elements (SP-1, CREB, GRE and distal C/EBP) far upstream of the first exon may be involved in the pathogenesis of NIDDM or obesity, and further studies need to be carried out.

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References


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