Human Obese Gene

Molecular Screening in Japanese and Asian Indian NIDDM Patients Associated With Obesity

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The mouse obese (ob) gene has recently been isolated through the positional cloning technique and has been proven to result in the obese and NIDDM phenotype in mice when mutated (Nature 372:425–432, 1994). More recently, it has been demonstrated, by experiments with recombinant ob protein, that ob gene product can cause mice, including ob/ob mice, diet-induced obesity mice, and normal mice, to lower their food intake and body weight (Science 269:540–549, 1995). To investigate the genetic and/or environmental influences underlying the development of NIDDM associated with obesity, we isolated and partially sequenced the human obese (OB) gene. The human OB gene isolated in this study encoded 167 amino acids and its open reading frame was revealed to be divided into two parts with an intermediate intron of ~2.4 kb. Using the single-strand conformation polymorphism (SSCP) technique, we screened Japanese and Asian Indian subjects for mutations in the protein coding regions of the OB gene. A total of 75 NIDDM patients with obesity (54 Japanese and 21 Asian Indians), 40 NIDDM patients without obesity (34 Japanese and 6 Asian Indians), and 34 Japanese patients with simple obesity showed no abnormal SSCP patterns in either component of the coding sequences. These results suggested that mutations in the coding regions of the OB gene are not likely to be commonly identifiable and that there would likely be a kind of obesity-associated NIDDM not caused by mutations of the OB gene. Diabetes 45:675–678, 1996

NIDDM is a heterogeneous disorder, and both genetic and environmental factors are involved in its pathogenesis. Several candidate genes, including genes for insulin (1), the insulin receptor (2), glucokinase (3), and mitochondrial tRNA Leu(UUR) gene (4), have been reported to contribute to the development of NIDDM. On the other hand, weight gain (5), diminished exercise (6), and antihypertensive medications (7) have also been suggested to affect the predisposition to development of NIDDM.

Obesity is usually found to be associated with a number of diseases, such as coronary heart disease and metabolic disorders (8,9), and is considered to be one of the most crucial factors affecting the development of NIDDM (10). Several abnormal conditions, such as impaired ability of insulin to suppress hepatic glucose production and to stimulate glucose disposal in muscle (11), peripheral insulin resistance and the resultant hyperinsulinemia and decrease in the number of cellular insulin receptors (12,13), and a deterioration in glucose-stimulated insulin secretion (14), are commonly observed in obese subjects. However, the mechanism by which obesity increases the risk for the development of NIDDM remains largely unknown.

The ob/ob mouse has been studied as one of the genetic rodent models in which both obesity and NIDDM are strongly heritable. This model has characteristic phenotypes such as hyperphagia, hyperinsulinemia, hyperglycemia, and hepatic insulin resistance (15). In 1994, Zhang et al. (16) reported the positional cloning of the mouse obese (ob) gene, the mutation of which resulted in severe hereditary obesity and NIDDM in mice. Recently, three laboratories have independently reported that recombinant ob protein injected into ob/ob mice has not only caused a reduction of food intake and body weight but normalized the serum levels of insulin and glucose, which are much higher in nontreated ob/ob mice (17–19). We assumed that mutations in the human obese (OB) gene may possibly contribute to the pathogenesis of obesity-associated NIDDM.

In the present study, we report the isolation and partial sequences of the human OB gene. (The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession numbers D63518 and D63519.) We screened, using polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP) analysis, NIDDM subjects with obesity in a Japanese and an Asian Indian population for the mutation in OB gene.

RESEARCH DESIGN AND METHODS

Subjects. The 88 unrelated Japanese NIDDM patients (34 men and 54 women) analyzed in this study were divided into two groups by their BMIs. The subjects with BMIs >25.0 kg/m² were defined as having NIDDM with obesity and those with BMIs <25.0 kg/m² were classified as...
MOLECULAR SCREENING OF HUMAN \( \text{ob} \) GENE

### TABLE 1
Profiles of the patients

<table>
<thead>
<tr>
<th>Patient group</th>
<th>( n ) (M/F)</th>
<th>Age (years)</th>
<th>Age at onset of NIDDM (years)</th>
<th>BMI (kg/m(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIDDM with obesity</td>
<td>54 (20/34)</td>
<td>54.6 ± 16.0</td>
<td>42.4 ± 15.9</td>
<td>29.2 ± 4.0</td>
</tr>
<tr>
<td>NIDDM without obesity</td>
<td>34 (14/20)</td>
<td>52.6 ± 13.4</td>
<td>41.2 ± 12.3</td>
<td>21.8 ± 2.2</td>
</tr>
<tr>
<td>Simple obesity</td>
<td>34 (13/21)</td>
<td>47.7 ± 22.5</td>
<td>—</td>
<td>31.9 ± 5.1</td>
</tr>
<tr>
<td>Indian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIDDM with obesity</td>
<td>21 (10/11)</td>
<td>30.6 ± 3.4</td>
<td>25.9 ± 3.1</td>
<td>31.8 ± 3.9</td>
</tr>
<tr>
<td>NIDDM without obesity</td>
<td>6 (4/2)</td>
<td>30.2 ± 9.4</td>
<td>28.7 ± 10.1</td>
<td>22.2 ± 1.1</td>
</tr>
</tbody>
</table>

Data are means ± SD or n.

having NIDDM without obesity. Another group of 34 patients with simple obesity, whose BMIs were >25.0 kg/m\(^2\) and who were not morbidly obese, were also examined as control subjects. Detailed information on these three patient groups is summarized in Table 1. To analyze another ethnic group, we additionally screened 27 unrelated Asian Indian NIDDM patients (14 men and 13 women). Each genomic DNA from these subjects was extracted from patients’ peripheral blood leukocytes in India and directly sent to the laboratory in Japan. A total of 21 NIDDM patients with obesity (BMI ≥25.0 kg/m\(^2\)) and 6 NIDDM patients without obesity (BMI <25.0 kg/m\(^2\)) were screened. The profiles of these Asian Indian subjects are also summarized in Table 1. The diagnosis of diabetes in these subjects was based on World Health Organization criteria. All participating individuals were informed about the aim of the study and gave their informed consent. The investigations were conducted according to the guidelines expressed in the Declaration of Helsinki.

**Isolation of the mouse \( \text{ob} \) cDNA.** Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (20) from 3T3-L1 adipocytes cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Reverse transcription (RT)–PCR with oligonucleotide primers: 5’-GGAGAACCTCCGGGATCTTC-3’ (nucleotides 107-130 of mouse \( \text{ob} \) cDNA) were performed with the initial primer and for RT. PCR was performed by the standard protocol in a programmable heat block (ASTEC Program Temp Control System PC-700). A denaturation step at 94°C was followed by 30 cycles of PCR with 5 μg total RNA was performed using specific oligonucleotide primers, which were arranged to amplify the region encompassing the entire open reading frame of the mouse \( \text{ob} \) cDNA. The sequences of the primers were 5’-GGGGGATCCATGTGCTGGAGACCCCTGTG-3’ (corresponded to residues 107-135) for the upstream primer containing a BamHI site and 3’-CTACAATCGGGACTTACGACTCTTAAGTTT-5’ (residues 599-629) as the downstream primer and for RT. PCR was performed by the standard protocol in a programmable heat block (ASTEC Program Temp Control System PC-700). A denaturation step at 94°C was followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min), and primer extension (72°C, 2 min).

The secondary PCR was performed for the subcloning with the RT-PCR product as a template. For the second PCR we synthesized degenerate primers: 5’-GGGGGATCCATGTGCTGGAGACCCCTGTG-3’ (corresponded to residues 107-135) for the upstream primer containing a BamHI site and 3’-CTACAATCGGGACTTACGACTCTTAAGTTT-5’ (residues 599-629) as the downstream primer and for RT. PCR was performed by the standard protocol in a programmable heat block (ASTEC Program Temp Control System PC-700). After amplification, the PCR product was digested with BglII and XhoI and then digested with restrictive endonucleases EcoRI and BamHI, yielding three fragments of ~6, 3.4, and 1.9 kb, respectively. Southern blotting analysis with the mouse \( \text{ob} \) cDNA gave a strong signal only in the fragment of 3.4 kb, indicating the presence of coding regions in this fragment. Figure 1 illustrates the schematic representation of the entire protein coding regions of the isolated human \( \text{OB} \) gene. Sequence analysis of these three patient groups is summarized in Table 1.

### RESULTS

**Cloning of the human \( \text{OB} \) gene.** Performing RT-PCR with total RNA isolated from 3T3-L1 adipocytes as a template, we obtained entire coding regions of the mouse \( \text{ob} \) cDNA. The nucleotide sequences of the PCR product were determined in the phagemid pBluescript II KS(+) (Stratagene, La Jolla, CA) and sequenced by the dideoxynucleotide sequencing method (21). The mouse \( \text{ob} \) cDNA was then labeled and used to screen a human genomic DNA library. One positive clone was isolated among ~5 X 10 \(^5\) plaque-forming units. The human \( \text{OB} \) genomic DNA obtained was digested with \( \text{XhoI} \) and \( \text{BamHI} \), yielding three fragments of ~6, 3.4, and 1.9 kb, respectively. Southern blotting analysis with the mouse \( \text{ob} \) cDNA gave a strong signal only in the fragment of 3.4 kb, indicating the presence of coding regions in this fragment. The mouse \( \text{ob} \) cDNA was sequenced with an A.L.F. DNA Sequencing Kit using the AutoRead Sequencing Kit (Pharmacia, Piscataway, NJ). Specific primers were settled around the 5’ or 3' end of the aforementioned partially sequenced nucleotides. The repetition of this procedure defined the full length of the coding sequences.

**PCR-SSCP analysis.** PCR-SSCP analysis was performed according to the method described previously (24–26). Briefly, the specific oligonucleotide primers amplifying coding sequences of \( \text{OB} \) genomic DNA were synthesized as shown in Table 2. The primer sets were labeled with [γ-\(^{32}\)P]ATP (ICN, Irvine, CA) before PCR, and 30 cycles of PCR amplification were carried out with a standard condition with 50 ng of patients’ genomic DNA as a template. The resultant fragments were loaded onto 5% polyacrylamide gel, with or without 10% glycerol, and electrophoresed, either at room temperature or at 4°C.

### TABLE 2
Sequences of primer pairs for PCR-SSCP of the human \( \text{OB} \) gene

<table>
<thead>
<tr>
<th>Set</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5’-TGTCTTTCAGGGCCCAAGAACCC</td>
<td>5’-GGAGAACCTCCGGGATCTTC</td>
<td>215</td>
</tr>
<tr>
<td>B1</td>
<td>5’-ACATGCTGAGACCTTGGTTC</td>
<td>5’-AGGGGATCCATGTGCTGGAGACCC</td>
<td>250</td>
</tr>
<tr>
<td>B2</td>
<td>5’-GGGAGAACCTCCGGGATCTTC</td>
<td>5’-CTACAATCGGGACTTACGACTCTTAAGTTT</td>
<td>241</td>
</tr>
</tbody>
</table>

Primer sets B1 and B2 are synthesized to amplify the forward part and the rear part of coding region B, respectively, and cover the overlapping sequences of 53 bp within coding region B. The positions of these primers are also shown in Fig. 1.
FIG. 1. Schematic representation of the entire coding regions of the human OB gene. Xho I/BamHI-digested fragment of 3.4 kb included two components (□) of the entire protein-coding sequences with the intermediate intron of ~2.4 kb. This fragment also involved noncoding 5' and 3' sequences of 330 and 200 bp, respectively. The small arrows indicate the positions of the synthetic oligonucleotide primers for PCR-SSCP analysis. The names of these primers are noted in Table 2. -s, upstream primers; -as, downstream primers.

region B for convenience. The isolated fragment also contained noncoding 5' and 3' sequences of ~330 and 200 bp, respectively.

The OB gene described in this study encodes 167 amino acids, which are 100% identical with those reported previously (16, 27). As suggested in a recent study (28), obesity gene proteins from various species have two variants, which are distinguished by the presence of a single glutamine residue at the amino acid position 49. Our OB gene encompasses the longer one with the glutamine at this position. Thus, nucleotide sequences predicted from our human genomic DNA have 82.7% homology with those from mouse cdNA (16) and are 100% identical with the human cDNA reported by Considine et al. (27).

PCR-SSCP analysis of the human OB gene. Both parts of OB gene coding regions of 115 unrelated NIDDM patients, who consisted of 88 Japanese and 27 Asian Indians, were scanned for mutations using PCR-SSCP analysis. The specific oligonucleotide primers for PCR-SSCP were synthesized as shown in Table 2, and the positions of these primers are indicated in Fig. 1. Primer set A, the upstream primer of set B1, and the downstream primer of set B2 are arranged in the noncoding sequences. The downstream primer of set B1 and the upstream primer of set B2 are settled in the middle of coding region B and thus cover the overlapping sequences of 53 bp within coding region B. We divided coding region B into two partially overlapping sequences for PCR amplification in an attempt to increase the sensitivity of SSCP analysis. Expected length of the PCR products with these primer sets was 215 bp (set A), 250 bp (set B1), and 241 bp (set B2), respectively. Successful amplifications of the PCR primers with confirmed agarose gel electrophoresis and sequencing.

First, in screening the Japanese patients (54 NIDDM patients with obesity, 34 NIDDM patients without obesity, and 34 patients with simple obesity), we found no abnormally migrating bands on SSCP analysis with four conditions with or without 10% glycerol in the gel, either at room temperature or at 4°C. Since we suspected that two samples with set B2 amplification might migrate subtly lower, we subcloned each of these PCR products into pT7Blue T-Vector (Novagen, Madison, WI) and sequenced 10 clones, respectively. All clones of each sample were revealed to have normal nucleotide sequences. Subsequently, to search for OB gene mutations in another ethnic group, we screened 27 unrelated Asian Indian NIDDM patients (21 with obesity and 6 without obesity) with the same PCR-SSCP analysis as in Japanese study. These results indicate the absence of mutations in the coding regions of the human OB gene in these subjects.

DISCUSSION

In this study, we reported the isolation of the human OB gene, and subsequently we successfully performed PCR-SSCP analysis of the entire protein-coding sequences of the OB gene in Japanese NIDDM and simple-obesity subjects and additionally in Asian Indian NIDDM patients. These establishments enable the molecular screening in human subjects of not only diabetes but also a number of genetic forms of obesity.

We detected no mutations in NIDDM subjects, regardless of the association with obesity, suggesting that mutation in the coding regions of the OB gene is not likely to be a commonly identifiable one that predisposes to development of NIDDM even when associated with obesity. SSCP variants were not identified in 34 Japanese subjects with simple obesity. This finding is consistent with the investigation in obese human subjects (27), which could discover neither a premature stop codon nor absence of the OB gene mRNA found in ob/ob mouse (16). In addition, other recent experiments have shown increased OB mRNA expression in fat tissue of obese humans, suggesting no existence of OB gene mutations and decreased sensitivity of obese humans to OB gene product (29, 30). These results presented here, however, do not exclude the possibility that mutations in the OB gene may contribute to obesity-associated NIDDM, particularly with a certain form of obesity, in some families or in other ethnic groups. It should also be noted that one of the reported ob mutations, which does not synthesize ob RNA in the co-isogenic SM/Ckc-+/Daco62J/o62J strain, is suggested to result from a structural alteration or sequence variation in the promoter region (16) that was missed in the present study. On the other hand, the db/db mouse, another genetic strain with an autosomal recessive inheritance, shows diabetes-associated phenotypes similar to the ob/ob strain (31). The phenotypes of this animal model are suggested to reflect a defect in the action of ob protein, possibly a receptor defect (32, 33). No reduction of food intake or body weight in db/db mice treated with recombinant ob protein (17–19) supports this hypothesis, raising the possibility that the gene encoding db protein may become another candidate for obesity-associated diabetes. In this context, further studies would be required for the characterization and screening of the promoter region of OB gene and for isolation of the ob receptor (presumably db) gene. Finally, during the revision process, Issel et al. (34) have reported the structural organization of the human OB gene. Taking our results and this recent observation into consideration together, our coding region A is encompassed in exon 2 and coding region B is settled in exon 3 of the human OB gene.

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REFERENCES