BRIEF REPORT

Biologically Inactive Leptin and Early-Onset Extreme Obesity

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SUMMARY

Mutations in the gene encoding leptin (*LEP*) typically lead to an absence of circulating leptin and to extreme obesity. We describe a 2-year-old boy with early-onset extreme obesity due to a novel homozygous transversion (c.298G→T) in *LEP*, leading to a change from aspartic acid to tyrosine at amino acid position 100 (p.D100Y) and high immunoreactive levels of leptin. Overexpression studies confirmed that the mutant protein is secreted but neither binds to nor activates the leptin receptor. The mutant protein failed to reduce food intake and body weight in leptin-deficient *ob/ob* mice. Treatment of the patient with recombinant human leptin (metreleptin) rapidly normalized eating behavior and resulted in weight loss.

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Congenital leptin deficiency is a very rare cause of early-onset extreme obesity.[1] It was first described in two extremely obese cousins from a consanguineous Pakistani family.[2] The conditions of both children were characterized by the absence of leptin in the circulation as a result of a homozygous frameshift mutation in *LEP*. Seven additional mutations have since been reported.[3-8]

The type I cytokine leptin is mainly produced by adipocytes to signal the energy state of the body and exerts its function as a satiety signal in the hypothalamus.[9] Clinical hallmarks of congenital leptin deficiency include early-onset extreme obesity, marked hyperphagia, and hormonal as well as metabolic disturbances.[2,3,10-12] Some patients have immunologic alterations.[11,13] Hormone replacement by means of daily injection of human recombinant metreleptin leads to rapid reductions in hyperphagia, fat mass, and metabolic and endocrine abnormalities.[10,12,13]

To date, reported cases of congenital leptin deficiency have been characterized by circulating leptin levels that are undetectable or very low as a result of defects in either synthesis or secretion of leptin.[2,3,5-8] Likewise, the corresponding *ob/ob* mouse model lacks leptin in the circulation.[9] However, a novel mutation in the murine gene encoding leptin (p.V145E) was generated by means of *N*-ethyl-*N*-nitrosourea mutagenesis.[14] The affected animals have hyperphagia and extreme obesity but also have marked hyperleptinemia, which suggests that the mutant leptin is biologically inactive.[14] Whether such congenital leptin dysfunction could also occur in humans has been unclear. Here we describe a case of early-onset extreme obesity due to a novel mutation in the leptin gene, accompanied by production and secretion of a mutant protein that is biologically inactive.

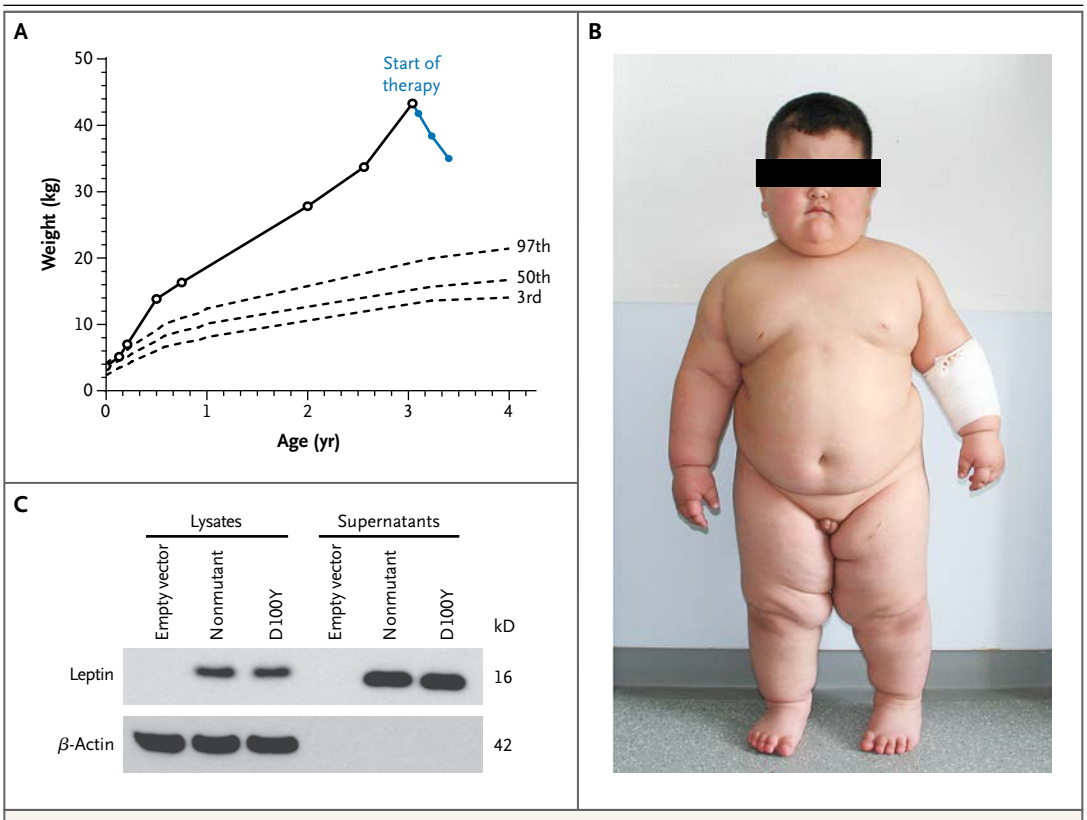

Figure 1. A Boy with a Novel Mutation in the Leptin Gene.

Panel A shows the weight curve for the patient, as compared with normal percentiles for boys of his age, before and after the start of treatment with metreleptin. Panel B is a photograph of the patient at the age of 2 years 6 months. Panel C shows the results after human embryonic kidney 293 (HEK293) cells were transiently transfected with empty vector or vector encoding nonmutant or D100Y leptin. After 48 hours, culture medium was collected and protein lysates were prepared. Leptin immunoreactivity was examined by Western blot analysis in cell lysates or medium supernatants, with β-actin serving as a loading control. The results of one representative experiment out of three performed are shown.

METHODS

CASE REPORT

The patient is the first child of two healthy, normal-weight Turkish parents with known consanguinity (first-degree cousins). He was born at 40 weeks of gestation with normal birth weight (3680 g); rapid weight gain started in the postnatal period (Fig. 1A and 1B). At presentation, the boy was 2 years 6 months of age and weighed 33.7 kg (>99.9th percentile; z score, 7.2), with a height of 93.5 cm (69.1st percentile; z score, 0.5). His body-mass index (the weight in kilograms divided by the square of the height in meters) was 38.6 (>99.9th percentile; z score, 5.8). Food-seeking behavior was reported by the parents (see the Supplementary Appendix, available with the full text of this article at NEJM.org, for a detailed phenotype description), and hyperphagia was proved in an ad libitum test breakfast in which the patient rapidly consumed a total of 680 kcal. The patient had recurrent ear and pulmonary infections requiring two intensive-care hospitalizations for severe pneumonia. Nevertheless, blood T-lymphocyte counts (3761 per cubic millimeter), subpopulation ratio ($CD4^+$–$CD8^+$ ratio, 1.7; reference range, 1.0 to 2.6), and function were normal, as indicated in Tables S1 and S2 in the Supplementary Appendix. Clinical and laboratory data are shown in Table 1.

DNA SEQUENCING AND LEPTIN MEASUREMENT

LEP sequencing was performed on genomic DNA with the use of standard protocols. The controls

Table 1. Characteristics of the Patient before and after the Start of Treatment with Metreleptin.*

Characteristic†	Week −120	Week −25	Week 0	Week 1	Week 3	Week 9	Week 18
Age (mo)	9	31	37	37	37	39	41
Weight (kg)	16.3	33.7	43.3	43	41.8	38.4	35.0
Body-mass index	30.2	38.5	44.6	44.3	42.6	39.2	34.2
Body-mass index z score	5.6	5.8	5.9	5.8	5.7	5.4	5.0
Fat mass (kg)	ND	ND	23.1	ND	ND	20.5	ND
Serum concentration							
Leptin (ng/ml)‡	32.5	42.6	48.7	59.6	32.8	20.0	14.5
Insulin (mU/liter)	ND	ND	19.2	10.6	9.7	10.8	4.8
C-peptide (ng/ml)	ND	ND	3.7	3.0	2.9	2.7	1.7
Triglycerides (mmol/liter)	ND	2.0	2.0	ND	ND	0.7	0.4
IGF-1 (ng/ml)	ND	<25	36	ND	ND	47	57
IGFBP-3 (ng/ml)	ND	1600	2360	ND	ND	3050	2530
Luteinizing hormone (U/liter)	ND	<0.10	<0.10	ND	ND	0.14	ND
Follicle-stimulating hormone (U/liter)	ND	0.93	0.79	ND	ND	1.39	ND
Aspartate aminotransferase (U/liter)	ND	70	110	ND	ND	43	ND
Alanine aminotransferase (U/liter)	ND	107	130	ND	ND	148	36

* To convert values for triglycerides to milligrams per deciliter, divide by 0.01129. IGF-1 denotes insulin-like growth factor 1, IGFBP-3 insulin-like growth factor binding protein 3, and ND not determined.
† The body-mass index is the weight in kilograms divided by the square of the height in meters.
‡ The first leptin measurement was performed with a commercial kit supplied by Mediagnost; all other leptin measurements were performed with a commercial kit supplied by IBL.

for genotyping were 720 schoolchildren from Ulm, Germany (146 of whom were of Turkish ancestry). Leptin concentrations in serum and cell culture medium were measured with enzyme-linked immunosorbent assays. Written informed consent was obtained from the parents of the patient and the controls. The study was approved by the ethics committee of the University of Ulm. For detailed information about the study methods, see the Supplementary Appendix.

CLONING AND SECRETION STUDIES

Nonmutant and mutant (D100Y) leptin was transiently expressed in human embryonic kidney 293 (HEK293) cells. Culture medium and cell lysates were subjected to Western blot analysis with a polyclonal leptin antibody (Biovendor). Nonmutant leptin-mCherry and mutant D100Y leptin-mCherry fluorescent fusion proteins were produced to study leptin receptor binding and internalization.

FUNCTIONAL STUDIES

HEK293 cells expressing the human leptin receptor (pMET7-hLR-FLAG) were generated. Detailed information on activity, binding, internalization, and competition studies is provided in the Supplementary Appendix.

PRODUCTION AND PURIFICATION OF RECOMBINANT LEPTIN PROTEINS

Nonmutant and D100Y leptin were produced in baculovirus-infected *Trichoplusia ni* BTI-Tn-5B1-4 cells (High Five Cell Line, Life Technologies). The proteins were purified from the corresponding cell supernatants by means of sequential chromatography.

STUDIES IN ANIMALS

Eight-week-old female *ob*/*ob* mice received daily intraperitoneal injections of vehicle (six mice), 0.2 μg of nonmutant leptin per gram of body weight (six mice), or 0.2 μg of D100Y leptin per gram (six mice), for 4 days. The dose was then increased to 0.6 μg per gram for 3 days. Body weight and food intake were monitored daily. A repeated-measurement two-way analysis of variance with Bonferroni-corrected multiple comparisons was performed with the use of GraphPad Prism software, version 6.01 (www.graphpad.com).

The experiments were approved by the local authorities.

RESULTS

LEP MUTATION

The serum level of leptin in our patient appeared to be high (42.6 ng per milliliter) (Table 1). On the basis of the clinical phenotype, we suspected a mutation in the gene encoding the leptin receptor (*LEPR*), which we ruled out by sequencing. Subsequent sequencing of *LEP* revealed a novel homozygous transversion (c.298G→T) in exon 3, leading to a change from aspartic acid to tyrosine at position 100 of the protein (p.D100Y) (Fig. S1 in the Supplementary Appendix). Both parents were heterozygous carriers of the mutation (Fig. S1 in the Supplementary Appendix). Screening of 720 ethnically similar children in southern Germany did not reveal any carriers of the mutation. It is not listed in dbSNP, the Human Gene Mutation Database, ClinVar, or the Ensembl Genome Browser.

All cases of congenital leptin deficiency to date have been characterized by an absence or near absence of circulating leptin (see the Supplementary Appendix for more information),[2,3,5-8] whereas our patient had high levels of the hormone. Therefore, we studied the secretory behavior of the D100Y mutant in a heterologous cell system. Plasmids encoding nonmutant and mutant leptin were transiently introduced into HEK293 cells. Both nonmutant and mutant proteins were abundantly present in cell lysates and were also detected in the medium supernatants of intact cells (Fig. 1C). This clearly shows that the novel mutation interferes neither with protein expression nor with secretion of leptin.

FUNCTIONAL ASSESSMENT OF MUTANT LEPTIN

To determine whether the D100Y mutant leptin is nonfunctional, we generated HEK293 cells with transient overexpression of the human leptin receptor (Fig. S2 in the Supplementary Appendix). These cells were treated with medium supernatants collected from cells overexpressing nonmutant and mutant leptin (adjusted to 30 ng of leptin per milliliter). Nonmutant leptin robustly induced the phosphorylation of Stat3 (Fig. 2A), which is typically activated on the binding of leptin to its receptor.[15] In contrast, the D100Y mutant failed to induce the phosphorylation of Stat3 (Fig. 2A). This finding indicates that the mutant D100Y leptin is nonfunctional.

The aspartic acid at position 100 resides at the beginning of helix C of the leptin protein.[16] Modeling of the 3D structure of the protein revealed that the side chain of mutant Y100 extends into the ligand binding pocket of the receptor (Fig. S3 in the Supplementary Appendix). This suggests that the mutant protein is unable to bind to the leptin receptor. To test this hypothesis, we generated fluorescence-labeled leptin. Whereas nonmutant leptin-mCherry was bound to leptin receptor at the membrane and internalized, D100Y leptin-mCherry was not (Fig. 2B).

To show that D100Y leptin is not functional in vivo, we studied leptin-deficient *ob/ob* mice. Daily intraperitoneal injections of 0.2 μg of nonmutant leptin per gram of body weight for 4 days resulted in significantly reduced food intake (Fig. 2C) and body weight (Fig. 2D) in 8-week-old female mice. In contrast, the mutant D100Y leptin was ineffective at the same dose and even when the dose was tripled, to 0.6 μg per gram of body weight, for 3 additional days (Fig. 2). This finding attests to the fact that the D100Y leptin is not functional in vivo.

TREATMENT WITH METRELEPTIN

Our patient had high circulating levels of mutant leptin. To determine whether these high endogenous levels could interfere with exogenous supplementation with metreleptin, we perfomed a competition assay in which leptin receptor–overexpressing cells were treated with increasing concentrations of nonmutant leptin (3 to 1000 ng per milliliter) in the absence and presence of high concentrations of recombinant D100Y leptin (100 and 300 ng per milliliter). Figure S5 in the Supplementary Appendix shows that there is no competition of nonmutant and mutant protein for leptin receptor activation, which is consistent with the notion that the mutant is unable to bind to the receptor (Fig. 2B).

We obtained written informed consent from our patient's parents to treat him with metreleptin. An initial dose of 0.03 mg of metreleptin per kilogram of lean body weight per day was chosen on the basis of previous experience with young patients with congenital leptin deficiency.[13] Subcutaneous metreleptin administration in our patient resulted in a rapid change in eating behavior, a reduction in daily energy intake, and substantial

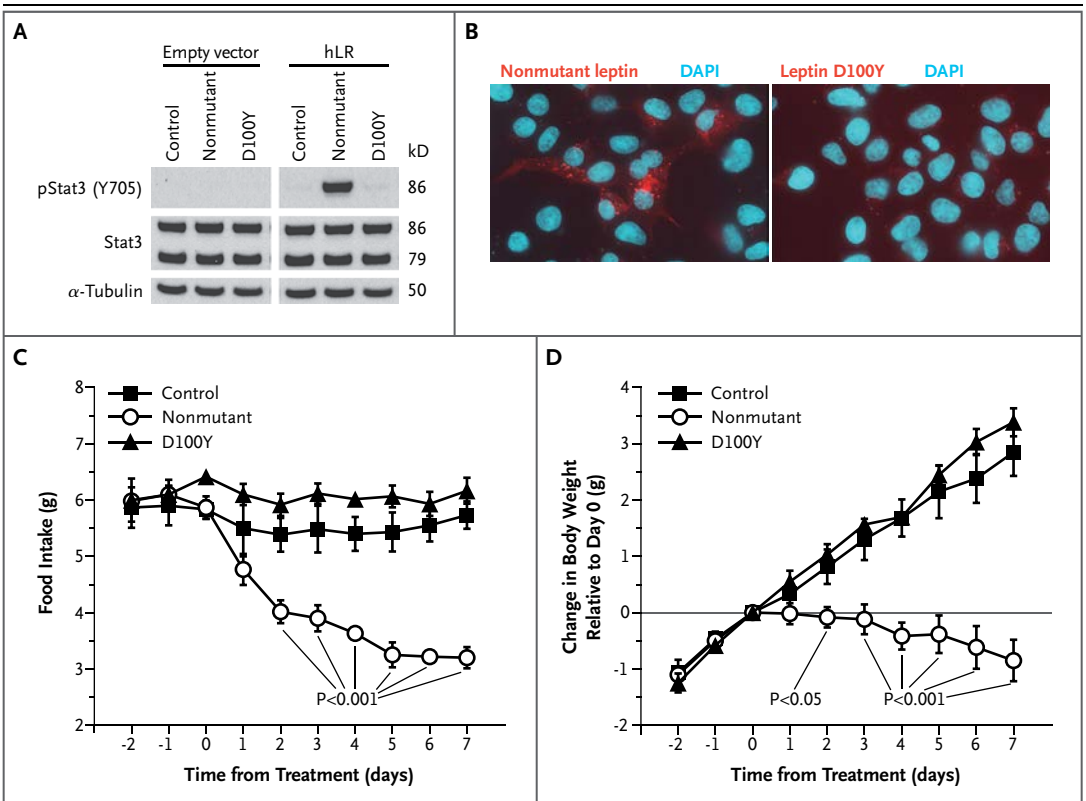

Figure 2. Biologic Inactivity of D100Y Mutated Leptin in Vitro and in Vivo.

Panel A shows the results of in vitro experiments demonstrating the absence of phosphorylation of Stat3 by the mutated D100Y leptin. HEK293 cells were transiently transfected with empty vector or vector encoding the human leptin receptor (hLR). Cells were treated with medium supernatants from HEK293 cells, which were transfected with empty vector (control), vector encoding nonmutant leptin, or D100Y leptin. Concentrations of nonmutant and D100Y leptin were adjusted to 30 ng per milliliter. After 15 minutes, protein lysates were prepared and subjected to Western blot analysis with pStat3 and Stat3 antibodies; α-tubulin served as a loading control. The results of one representative experiment of the three performed are shown. Panel B shows the results of experiments demonstrating the inability of D100Y leptin to bind to the leptin receptor. HEK293 cells were transiently transfected with empty vector or vector encoding hLR and then treated with mCherry-labeled nonmutant or D100Y leptin for 30 minutes. The cells were subsequently analyzed with the use of fluorescence microscopy and staining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI). The results of one representative experiment of the three performed are shown. Panels C and D show the results of the experiments in mice. Female 8-week-old *ob/ob* mice received daily intraperitoneal injections of wild-type or D100Y leptin or vehicle at a dose of 0.2 μg per gram of body weight for 4 days (six mice per group). The dose was then increased to 0.6 μg per gram for an additional 3 days. Changes in food intake and body weight were monitored daily. Data points are means, and I bars indicate standard errors. A repeated-measurement two-way analysis of variance with Bonferroni-corrected multiple comparisons was performed. Adjusted P values are for the comparison with control values at the corresponding point in time.

weight loss, as well as in metabolic and hormonal changes (Fig. 1A and Table 1).

DISCUSSION

Current clinical recommendations advise that leptin serum concentrations be measured in children who have rapid weight gain in the first months of life, to identify patients with congenital leptin deficiency.[17] These recommendations are supported by the fact that all described cases of congenital leptin deficiency have been characterized by undetectable or very low levels of leptin in the circulation.[2,3,5-8] Corroborating these clinical observations, studies in heterologous cell systems have shown that the previously described *LEP* mutations lead to defects in leptin synthesis or secretion.[2,3,5-7]

We describe a case of early-onset, extreme obesity caused by a biologically inactive mutant leptin that was present at high levels in the circulation. The p.D100Y amino acid change results from a previously unknown homozygous transversion (c.298G→T) in exon 3 of *LEP*. Using HEK293 cells, we demonstrated that the mutant hormone is secreted into the cell culture medium in a way that is similar to secretion of nonmutant leptin, which is in line with the serum measurements. The circulating leptin levels in our patient were within the range of levels found in other children with extreme obesity not related to leptin deficiency.[7] The secreted amounts of mutant leptin therefore seem to correspond to the patient's body fat mass.

The clear clinical phenotype of our patient, characterized by food-seeking behavior, hyperphagia, and extreme obesity, led us to the hypothesis that the mutant leptin was not functional and therefore was unable to mediate a satiety signal in the central nervous system. There are several pieces of evidence to support this hypothesis. First, whereas nonmutant leptin clearly led to phosphorylation of Stat3 in leptin receptor–overexpressing HEK293 cells, the mutant protein was unable to induce this effect. Second, the mutant protein did not bind to the leptin receptor, whereas nonmutant leptin interacted with its receptor at the cell surface and was internalized into the cells. Third, administration of D100Y mutant leptin did not control food intake or body weight in *ob/ob* mice, whereas nonmutant leptin clearly exerted these well-known functions in vivo.[18] This set of data indicates that the D100Y leptin mutant is not functional.

Finally, treatment of the patient with metreleptin was successful, resulting in a rapid change in eating behavior and significant weight loss. The circulating levels of leptin progressively decreased during therapy with metreleptin, which probably reflected the negative energy balance as well as down-regulation of endogenously produced hormone on the level of the adipocyte due to a known negative feedback loop.[19]

Mutations leading to biologically inactive hormones are rare. The phenomenon of bioinactivity has been observed for other protein hormones, such as corticotropin,[20] thyrotropin,[21] and growth hormone.[22] In these examples, single amino acid substitutions give rise to protein hormones that display immunoreactivity but lack biologic activity, which illustrates the limitations of immunoassay-based diagnostic tests. The majority of cases of hormone deficiency are not related to bioinactivity but rather to a defect in hormone production or secretion. In the case of congenital leptin deficiency, eight disease-causing mutations have been identified[2,3,5-8] that result in protein misfolding (e.g., because formation of the intracellular disulfide bond is hindered), aberrant intracellular transport, or defective secretion.[2,23,24] Nonsense-mediated messenger RNA decay after insertion of a premature stop codon has also been proposed as a mechanism leading to hormone deficiency.[2]

In summary, our patient has congenital leptin deficiency due to a biologically inactive leptin associated with high circulating hormone levels. Given our findings, circulating levels of the hormone that appear to be normal in relation to body-mass index and fat mass do not rule out disease-causing mutations in the gene encoding leptin and might obscure the correct diagnosis.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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