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Amino Acid Variants in the Human Leptin Receptor: Lack of Association to Juvenile Onset Obesity

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The recently described putative lipostat system mediated in part by leptin and its hypothalamic receptor provides logical candidate genes for the molecular basis of inherited obesity in humans on the basis of the occurrence of profound obesity observed in obese and diabetic mice, in which the genes for leptin or its receptor, respectively, are mutated. In this study we tested the hypothesis that juvenile onset obesity in humans may be caused by leptin resistance mediated through genetic variations in isoforms of the hypothalamic leptin receptor. One hundred and fifty-six obese Danish men with a history of juvenile onset obesity were selected at the draft board examination with a body mass index (BMI) $\geq$31 kg/m^2. From the same study population a control group of 205 control subjects (mean BMI = 21,5 kg/m^2) were randomly selected. Single strand conformational polymorphism scanning of genomic DNA from 56 obese subjects revealed a total of four amino acid variants located in coding exons 2, (Lys109Arg), 4 (Lys204Arg and Gln223Arg), and 12 (Lys656Asn), respectively. The codons 109, 223, and 656 variants were common, but their prevalence was not significantly different between obese and lean carriers with regard to allele or carrier frequency ($p > 0.1$ in each case). The codon 204 mutation was only found in one obese subject. In conclusion, it is unlikely that mutations in the coding region of the long isoform of the leptin receptor are a common cause of juvenile onset obesity.

Obesity is a common disorder affecting Westernised societies and it reflects a combination of genetic and environmental influences. Studies of twins (1,2), as well as adopted children (3-5) show that most, if not all, familial aggregation of obesity is due to genetic influence rather than to shared family environment. A major obstacle in rational treatment of obesity is the inability to sustain weight loss. Studies suggest the existence of an inherent "setpoint" of adiposity, with perturbations of adiposity opposed by increasing or decreasing energy expenditure (6). Such a putative setpoint might be determined in part by genes, and thus may constitute the mechanism of inherited obesity. The recently described putative lipostat system mediated by leptin and its hypothalamic receptor (7,8) constitutes a qualified candidate for the molecular basis of this mechanism (9).

Cloning of a leptin receptor has been reported in several papers (8,10,11), all describing common extracellular and transmembrane sequences and several splice variants of the intracellular domain, including a putative soluble receptor form. However, intracellular signalling through the receptor is thought to be mediated exclusively by one long splice form of the receptor containing putative JAK and STAT signalling motifs (8). The long isoform is in mice primarily expressed in the hypothalamic region (8) and its importance in mediating leptin signalling is evident in the *diabetes* (Lepr^{db}/Lepr^{db}) mouse where a single base substitution causes erroneous splicing and truncation of the long isoform, leading to severe obesity identical to the *obese* (Lep^{ob}/Lep^{ob}) mouse which completely lacks the receptor ligand, leptin (7,10,12). We have previously analysed the human homologue of the leptin gene for mutations in juvenile onset human obesity and foumd one missense mutation, whose influence could not be determined (13).

In this study we report the results of single strand conformational polymorphism (SSCP) scanning of the long isoform of the leptin receptor as well as the putative truncated receptor in subjects with juvenile onset obesity. We also analysed the prevalence of coding region variants in 156 subjects with juvenile onset obesity and in a control group of 205 subjects.

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TABLE 1

BMI Values in kg/m^2 of Obese and Control Subjects at Age 7, 13, 20, 37, and 48

Age (years)	BMI (kg/m^2)			
	Obese		Control	
	n	Mean ± SD	n	Mean ± SD
7	147[a]	18,4 ± 2,4	185[a]	15,8 ± 1,2
13	148[a]	25,1 ± 3,2	177[a]	18,6 ± 2,2
20*	156	33,4 ± 2,6	205	21,5 ± 2,2
37*	156	34,0 ± 5,1	205	24,9 ± 3,2
48*	156	35,4 ± 5,5	205	26,4 ± 3,9

* Average age.
[a] BMI values were not available for all subjects.

SUBJECTS

The study population consists of young Danish men, who around age 20 (range 18-26 yr) were examined at the draft board, and who, in addition, had attended school in the Copenhagen municipality, where height and weight were measured as a part of the school health examination (14). The sample was further restricted to those who were examined at the Copenhagen City Heart Study Programme in 1981-83 (15) and again in 1992-94. The study group of men with juvenile onset obesity were the 156 who had a BMI $\geqslant$ 31 kg/m^2 at the draft board examination. A control group of 205 draftees were selected at random as every hundredth from the same study population. Weight and height at different ages were recorded and BMI values at ages 7, 13 and on average 20, 37 and 48 years are listed in Table 1. Genomic DNA was obtained from blood samples, drawn at the last examination at the Copenhagen City Heart Study. A subgroup of 56 obese subjects were randomly selected from the obese group for the initial SSCP scanning. The study was approved by the Ethical Committee of Copenhagen and was in accordance with the principles of the declaration of Helsinki.

METHODS

DNA isolation. Genomic DNA was isolated by a method adapted from Miller et al (16). Human leukocyte nuclei from whole blood were precipitated, washed and lysed in 0.5% SDS and Proteinase K. Proteins were precipitated by addition of a saturated NaCl solution, and genomic DNA was subsequently precipitated by ethanol precipitation.

PCR-SSCP and heteroduplex analysis. Combined SSCP and heteroduplex analysis was performed on 56 subjects with juvenile onset obesity, randomly selected from the obese group. From the genomic sequence, primer pairs for PCR-SSCP specific to the genomic sequence of each exon were synthesised as shown in Table 2. The primer and gene sequence was obtained from GeneBank (Accession: SEG_HSOBRI) and from Leibel et al. (17). Coding exons 1 to 17 encode the common extracellular and transmembrane region of the different leptin receptor splice isoforms. Coding exon 14 encodes the terminal exon of a putative truncated receptor isoform, similar to the Re receptor form in mice (10). Coding exon 18 encodes the terminal exon of the long isoform of the receptor. Exons 2, 4, 7 and 18 were split in two or more PCR segments.

DNA segments were PCR amplified and analysed for SSCP and heteroduplex formation by non-denaturing gel electrophoresis at 2 different conditions (5% glycerol at 25°C and 1% glycerol at 6-8°C), as described previously (13,18). Using segments of around 250 bp this method in our laboratory has a sensitivity of greater than 90% in detecting known mutations. PCR was carried out in a volume of 25 μl, using 100 ng DNA with 5 pmol specific primers and 1.5 mmol/l $MgCl_2$ and including 0.125 μl [α-P^{32}]dCTP (3,000 Ci/mmol; 1 Ci = 37 Gbq; Amersham) and 0.5 U Taq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT). Samples were subjected to the following PCR conditions; initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C or 52°C for 30 sec and extension at 72°C for 30 sec, terminated by extension at 72°C for 5 min. Cycling was performed in thin-walled tubes on a GeneAmp 9600 Thermocycler (Perkin-Elmer/Cetus, Norwalk, CT).

Samples were mixed 1:5 with loading buffer (90% formamide, 10% NaOH, xylencyanol and bromophenol blue), denatured and allowed to partially re-anneal prior to loading to generate heteroduplexes. Gels were exposed on X-ray film overnight at-70°C, and gels were analysed for variations in migration. All variant alleles were re-amplified by PCR and sequenced both directions using the Thermo-Sequenase kit (Amersham, Buckingham PLC, UK).

Mutation screening methods. The codon 204 variant creates a recognition site for the restriction enzyme *Hinf I*. Screening for this variant was performed by PCR amplifying exon 4 segments using primers 4Ia and 4IIb, and adding one unit of *Hinf I* directly into the product, and after incubation, analysing on a 3% agarose gel. Similarly the codon 223 variant creates a recognition site for the restriction enzyme *Msp I* in the same PCR segment, and this variant was detected by adding 1 unit of *Msp I* enzyme to PCR amplified exon 4 segments as described above. Codons 109 and 656 variants were screened by SSCP.

Statistical methods. Comparisons between groups were evaluated by Chi square test. Statistical Package of Social Science for Windows, version 6.10, was used for statistical analysis.

RESULTS

SSCP scanning. SSCP scanning of the entire long isoform of the leptin receptor revealed a total of 8 sequence variants, listed in Table 3. Four amino acid variants were identified in coding exons 2, (Lys-109Arg), 4 (Lys204Arg and Gln223Arg) and 12 (Lys-656Asn), respectively. The prevalences of these four variants were determined in the cohorts of 156 obese and 205 control subjects and the results are given in Table 3. The prevalences of mutations were similar in the two cohorts for all three frequent variants ($p = 0.64$, $p = 0.52$ and $p = 0.94$ for exons 2, 4 and 12 variants, respectively), and the distributions of the alleles were in Hardy-Weinberg equilibrium (not shown). BMI values of homozygous and heterozygous carriers of the variants at different ages are listed in Table 4. Stratifying the cohort of control subjects into a group with BMI below 25 kg/m^2 at the draft board examination ($n = 190$) did not demonstrate any increased prevalence of leptin receptor variants when compared to the 156 obese subjects. The codon 204 variant was only identified in one obese subject, whose BMI was not different from the mean value of the obese group (Table 4). Summarising the variants and testing for the combined effect of mutations in the two cohorts did not show any association to obesity. However, this analysis is confounded by the fact that the coding variants for exons 4 and 12 are in apparent linkage disequilibrium, since

TABLE 2

Primers for PCR SSCP Analysis of the Human Leptin Receptor on Genomic DNA

Exon	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	PCR primer annealing temperature
1	GCT-ATT-GGA-CTG-ACT-TTT-CTT-AT	GGC-TTC-ACT-AAC-TCA-CTG-GTT-AGA-AC	55°C
2I	TCA-GAT-ACT-TTC-TAT-TCA-TGT-CTT-AGT	ATA-AGT-TAG-AAA-AGT-GAG-TAC-CAC-TTG	55°C
2II	AAT-TCG-AAT-GGA-CAT-TAT-GA	GTA-TCA-AAG-AAT-TAA-AAA-ACA-TTG-TTC	55°C
3	CTT-AAA-TTT-TTT-TTT-TTT-TTT-TGT-TTT-TTT-T	TAA-AAA-AAC-TGT-GTA-TTA-GAA-ATG-C	52°C
4I	TCC-TCT-TTA-AAA-GCC-TAT-CCA-GTA-TTT	ACA-TAA-GGA-GAG-TGT-CGT	55°C
4II	GTG-TTC-ATG-AAT-GTT-GTG-AAT	AGC-TAG-CAA-ATA-TTT-TTG-TAA-GCA-AT	55°C
5	GAC-TTT-ATT-TTA-TTC-AGC-TAT-AAT-TGT	GCA-GAG-GGT-AAT-TGC-TAT-GGG-AC	55°C
6	AGT-AAC-GGT-TCC-AGA-TCA-ACT-TG	GGC-CTC-AAA-ATG-TAA-GAT-GCT-TAT-AC	55°C
7I	GCA-GTG-TAA-CTT-CTG-AAT-GTG-TTG-T	AGT-ACA-CTG-CAT-CAT-AGG-TAA-ACT	55°C
7II	ATC-TAT-AAG-AAG-GAA-AAC-AAG-ATT-GT	GGA-CTC-TAA-TAT-AAG-GAG-GGG-TCC-AT	55°C
8	TAT-ATT-AGA-GTC-CTG-TTA-AAG-ATG	ATT-TTT-ATC-TCA-CTG-TGC-CCA-C	55°C
9	GAA-TTC-TCA-GAT-ATC-TTC-TTG-TTG-C	CAT-TAA-ATC-TGC-ATC-AAT-CTG-CAT-AC	55°C
10	CAC-AAT-GTT-TTT-AGG-CAT-TAT-TAC	TCT-AAT-GCA-ATT-AAA-CTC-TTA-CAT-ATT	55°C
11	GTA-CTT-CAG-GGC-CCT-TTA-GAT-ACA-TA	TTT-GAA-GAA-TAC-TTT-TCA-GCC-ATA	55°C
12	GCA-TAA-GTG-TGT-GCT-TCA-AAT-ATG-G	CGA-AGA-TTA-ATA-ACA-GGA-TTA-TGG-ACC	55°C
13	TCA-GTT-AGT-ATA-AAA-AGC-ACT-GCA-GC	TGC-AAA-AGT-TAA-ATA-TTA-AAA-GAG-GC	55°C
14	TAA-GTT-CCT-CAA-GAG-TAT-TAG-TAG	TTT-TGA-AGT-TTT-CAT-TAA-CTG-GC	55°C
15	ATG-TAT-GTT-CCA-CTC-ATT-ACT-ATT-A	CAA-TAT-TAC-TGC-AAA-CAA-ATT-AGG-CAC	55°C
16	GTG-ATG-AAT-TCA-GAA-AAT-GTC-TAC-T	AAT-CAG-GGT-TTG-AAT-ACG-CGT-A	55°C
17	ACT-AAC-TGT-TCA-CAT-TTT-CCA-ATA-TGG	ATA-ACA-GAT-ATA-TTA-AGA-TG	55°C
18I	GCA-AAA-TTT-TTT-AAC-ATA-ATT-GAG-CC	GCC-TCA-TAG-GTT-ACC-TCA-GTA-CC	55°C
18II	CAG-TTC-AAC-AGT-GTT-AAC-TTC-TCT-G	TGC-TGA-TCT-GAT-AAT-ATA-AAA-AAT-G	55°C
18III	AAT-AGC-TCA-TGG-GAG-ATA-GAG	GTC-TTC-TTA-CTA-GAA-GTT-CCT-AAG-TTG	55°C
18IV	GGA-CAG-TTG-CTC-ACA-CTT-TGT-AG	CAC-CCA-CAA-CTA-TAA-TCT-ATT-ACA-C	55°C

they do not appear to coexist on the same alleles. The prevalence of the intronic and non-coding variants were not determined in the entire cohorts.

DISCUSSION

The presence of receptors for leptin both in the choroid plexus, an important entry point to the brain of blood borne molecules, and in the hypothalamus, makes the various isoforms of the leptin receptor obvious candidates for genetic defects involved in leptin resistance (19,20).

Mutational analysis of the genomic structure of the human leptin receptor has not previously been reported. However, brain tissue-derived cDNA encoding the long isoform of the leptin receptor has been ana-

TABLE 3

Polymorphic Positions in the Human Leptin Receptor and Their Prevalence in 156 Subjects with Juvenile Onset Obesity and 205 Control Subjects

Coding exon	Position nucleotide #	Variant	Allele frequency (%)		Carrier frequency (%)	
			Obese	Control	Obese (ho/he)	Control (ho/he)
2	cDNA 326	K109R	25,1	23,8	7,0/35,0	7,0/34,0
4	cDNA 611	K204R	0,3	0,0	0,0/0,7	0,0/0,0
4	cDNA 668	E223R	46,0	39,1	19,5/49,2	15,2/48,2
12	cDNA 1968	K656N	17,0	15,0	3,6/26,6	1,6/28,0
Intron						
5	Intron + 49	A → G	15,0[a]	nd	2,0/26,0[a]	nd
7	Intron + 20	G → A	1.0[a]	nd	0,0/2,0[a]	nd
17	Intron + 37	C → A	58,0[a]	nd	12,0/34,0[a]	nd
3′UTR	cDNA 3056	G → A	37,5[a]	nd	15,4/44,2[a]	nd

nd, not determined ho, homozygous; he, heterozygous.
[a] Determined in 56 obese subjects.

TABLE 4

BMI Values (kg/m^2) of Obese Carriers of Leptin Receptor Coding Sequence Variants

	BMI at 7 yr.		BMI at 13 yr.		BMI at 20* yr.		BMI at 37* yr.		BMI at 48* yr	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
All obese	146[a]	18,4 ± 2,4	148[a]	25,1 ± 3,1	156	33,4 ± 2.6	156	34,0 ± 5,2	156	35,4 ± 5,5
K109R he	52	18,3 ± 2,3	52	24,5 ± 3,0	56	33,1 ± 1,9	56	33,0 ± 4,8	56	33,9 ± 4,9
K109R ho	9	18,8 ± 3,6	10	26,7 ± 2,2	10	33,0 ± 1,9	10	31,9 ± 5,5	10	36,0 ± 7,1
K204R he	1	18,2	1	26,3	1	31,1	1	33,2	1	35,0
E223R he	60	18,3 ± 2,0	61	25,2 ± 3,0	65	33,2 ± 2,6	65	33,9 ± 4,9	65	34,7 ± 5,2
E223R ho	26	18,2 ± 2,0	25	25,1 ± 2,7	27	33,7 ± 2,7	27	33,4 ± 5,0	27	34,8 ± 5,8
K656N he	33	18,9 ± 2,5	35	26,0 ± 3,6	37	34,2 ± 3,5	37	34,8 ± 5,8	37	36,8 ± 6,6
K656N ho	5	17,4 ± 2,1	5	23,5 ± 2,7	5	31,9 ± 0,6	5	33,5 ± 4,6	5	34,8 ± 5,1

* Average age.
[a] BMI values were not available for all subjects.
ho, homozygous; he, heterozygous.

lysed for mutations in 15 human subjects (8 obese, 7 lean), where two coding sequence polymorphisms were identified: a glutamine to arginine substitution at cDNA position 668, and a lysine to arginine substitution at cDNA position 326 (21) (in the original paper the codon 109 substitution was mistaken for a silent polymorphism and reported as such).

Using genomic DNA we performed mutational analysis of the long isoform of the leptin receptor on a selected group of 56 subjects with juvenile onset obesity.

In our analysis we have identified four amino acid variants, and additionally four silent polymorphisms. The prevalence of the amino acid variants were examined in 156 obese subjects and 205 control subjects. However, in our population, these sequence variants are not related to BMI at age 7, 13, 20, 37 or 48. Stratifying the control sample into a group with BMI < 25 kg/m^2 did not show further association. The amino acid variant in codon 204 was found only in one obese subject, and thus the significance of this variant cannot be evaluated from this association study. Segregational analysis of this variant with elevated BMI or serum leptin levels in the family of the carrier or in vitro studies will be necessary to examine for a possible functional effect of this mutation. No coding sequence variants have been identified in exon 14, which is specific to the putative soluble form of the leptin receptor, or in exon 18, encoding the terminal exon of the long isoform. Thus, the variants identified in exons 2, 4 and 12, are common to all known isoforms of the human leptin receptor.

In conclusion, it is unlikely that mutations in the coding region of the long isoform of the leptin receptor are a common cause of juvenile onset obesity.

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