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Diabetes. Author manuscript; available in PMC 2011 February 24.

Published in final edited form as:
Diabetes. 2004 November ; 53(11): 2992–2997.

Variation in *NCB5OR*:

Studies of Relationships to Type 2 Diabetes, Maturity-Onset Diabetes of the Young, and Gestational Diabetes Mellitus

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Abstract

Recent data show that homozygous *Ncb5or*$^{-/-}$ knockout mice present with an early-onset nonautoimmune diabetes phenotype. Furthermore, genome-wide scans have reported linkage to the chromosome 6q14.2 region close to the human *NCB5OR*. We therefore considered *NCB5OR* to be a biological and positional candidate gene and examined the coding region of *NCB5OR* in 120 type 2 diabetic patients and 63 patients with maturity-onset diabetes of the young using denaturing high-performance liquid chromatography. We identified a total of 22 novel nucleotide variants. Three variants [IVS5+7del(CT), Gln187Arg, and His223Arg] were genotyped in a case-control design comprising 1,246 subjects (717 type 2 diabetic patients and 529 subjects with normal glucose tolerance). In addition, four rare variants were investigated for cosegregation with diabetes in multiplex type 2 diabetic families. The IVS5+7del (CT) variant was associated with common late-onset type 2 diabetes; however, we failed to relate this variant to any diabetes-related quantitative traits among the 529 control subjects. Thus, variation in the coding region of *NCB5OR* is not a major contributor in the pathogenesis of nonautoimmune diabetes.

Type 2 diabetes is a common metabolic disease with a pathophysiology characterized by decreased insulin secretion and insulin resistance. There is substantial evidence for genetic components in the pathogenesis of the nonautoimmune forms of diabetes, specifically for the autosomal dominantly inherited maturity-onset diabetes of the young (MODY), where the impaired glucose regulation is due to a deficiency in pancreatic β-cell insulin secretion. Several subtypes of MODY are recognized as being caused by single heterozygous mutations in genes encoding various transcription factors: hepatocyte nuclear factors (HNFs), along with insulin promoting factor-1, neurogenic differentiation factor D, and the glycolytic enzyme glucokinase (GCK) (1–7). Mutations in the known MODY genes explain only ~50–70% of all MODY cases (8–10). Thus, other genes, so-called MODY-X genes, are needed to explain the remaining MODY cases.

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A targeted knock-out of the novel oxidoreductase Ncb5or in mice was recently reported in which it was shown that homozygous *Ncb5or*$^{-/-}$ mice developed a diabetic phenotype (11). The enzyme is a widely expressed cytosolic cytochrome *b*-type NAD(P)H oxidoreductase containing cytochrome *b*5 and *b5* reductase domains (12). Young *Ncb5or*$^{-/-}$ mice (aged 4 weeks) had impaired glucose tolerance and low insulin secretion, and after 7 weeks a considerable rise in blood glucose levels was observed when compared with *Ncb5or*$^{+/+}$ mice. *Ncb5or*$^{+/-}$ heterozygote mice had normal blood glucose levels and normal glucose tolerance (NGT). For older *Ncb5or*$^{-/-}$ mice there was a modest reduction in the number of islets, which in addition were distinctly depleted of β-cells. Furthermore, islets from 4-week-old *Ncb5or*$^{-/-}$ mice showed a 40% decrease in insulin content compared with size-matched islets from *Ncb5or*$^{+/+}$ mice. *Ncb5or*$^{-/-}$ mice at age 7 weeks also had elevated serum triglycerides and cholesterol levels and were hyperphagic (11).

Since absence of Ncb5or expression resulted in a diabetic phenotype with a noninflammatory β-cell deficiency, we hypothesized that variation in human NCB5OR might be related to MODY-X or type 2 diabetes. Furthermore, owing to the relatively low age at the development of the phenotype, we included various early-onset diabetes subtypes in this supposition. Indeed, in a genome-wide scan (13) of MODY-X families, a linkage peak was identified on chromosome 6 (flanked by markers D6S1017 and D6S460) close to the chromosomal localization of human *NCB5OR* (6q14.2). Moreover, a genome-wide scan of Finnish affected sibling pairs showed strong linkage of a region on chromosome 6 close to *NCB5OR* with late-onset type 2 diabetes and HDL/total cholesterol ratios (14). In this study we investigated the entire coding region of human *NCB5OR*, including intron-exon boundaries for variation using denaturing high-performance liquid chromatography (dHPLC) and subsequent nucleotide sequencing in 183 diabetic patients including probands from MODY-X families, early-onset type 2 diabetic patients, late-onset type 2 diabetic patients, and women diagnosed with gestational diabetes mellitus (GDM). A total of 22 variants were identified (Fig. 1). Three variants were further investigated by genotyping in a group of 717 type 2 diabetic patients and in a group of 529 control subjects with NGT. The Gln187Arg (identified in one patient) and His223Arg (in four patients) variants were chosen due to their potential impact on NCB5OR protein function and the IVS5+7del(CT) (one patient) variant because it is located near a predicted splice site and may therefore influence mRNA expression and/or stability. The most frequent variant was IVS14–72T>C with a minor allele frequency (MAF) of ~8% followed by IVS14–166C>T and IVS15–259C>A with approximate MAFs of 5% each. All other variants had MAFs of >3% or lower (estimated from the primary mutation analysis of 183 subjects). The degree of linkage disequilibrium between the variants was estimated. The IVS2–15T>C and IVS3+81G>A variants were in complete linkage disequilibrium. The IVS5–245A>G and IVS6+26A>G variants were also in complete linkage disequilibrium and furthermore in tight linkage disequilibrium with the IVS8–196A>G (R^2 = 0.8). There was no evidence of linkage between any of the remaining variants. This may be due to the relatively low allele frequencies of the variants.

Genotyping of the Gln187Arg variant did not result in the identification of any additional carriers among a total of 1,246 subjects. The patient for whom the variant was detected was a 49-year-old woman diagnosed with GDM during her second pregnancy at age 38 years. To examine a potential impact of the Gln187Arg variant on GDM, we genotyped a group involving 140 women with GDM; however, still no carriers of the variant were found. We carried out case-control studies for the His223Arg and IVS5+7del(CT) variants (Table 1). For the His223Arg variant we observed no differences between the diabetic patients and the control subjects for genotypic distribution or MAF. For the IVS5+7del(CT) variant the allele frequency was 0.3% (95% CI 0.0 – 0.6) for the type 2 diabetic patients and 1.0% (0.4–1.6) for the glucose-tolerant subjects. This difference was statistically significant (P = 0.03),

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which was also the case for the distribution of the genotype groups ($P = 0.03$, Table 1). For the diabetic patients, no differences were observed in age of onset, BMI, waist circumference, fasting serum C-peptide, or treatment between carriers and noncarriers of the IVS5+7del(CT) variant (data not shown).

For both the His223Arg and IVS5+7del(CT) variants we performed a study in the group of 529 glucose-tolerant subjects of the interaction between genotype groups and a range of quantitative traits related to type 2 diabetes and obesity (Table 2). We did not observe any significant associations for any of the phenotypes with the genotype group, and because there were no homozygous carriers, a tendency toward significant alterations in the metabolic variables was difficult to detect. One characteristic feature of the $Ncb5or^{-/-}$ knock-out mice was an increase in circulating triglyceride and cholesterol levels (11). We observed a lower fasting serum total cholesterol level among the 10 carriers of the IVS5+7del(CT) variant and the four His223Arg heterozygotes, although this was not significant (Table 2). In addition, the IVS5+7del(CT) carriers had slightly lower fasting serum triglyceride and insulin levels, although this did not reach statistical significance either.

Two single nucleotide polymorphisms (SNPs) identified in an in silico search (rs1408932 and rs10080628) were not observed among 1,246 participants, and we assume that they are artifacts from sequencing of the chromosome 6 clones.

Three intronic variants (IVS4 – 89A>T, IVS5–131T>C, and IVS6–59A>G) were identified in only one patient each. These patients were the probands of three MODY-X families, and although these variants are unlikely to have any functional impact on NCB5OR, they may be in linkage disequilibrium with a yet unidentified causative variant located outside the area included in the present mutation analysis. DNA was obtained from the remaining family members who were genotyped for the respective variants using nucleotide sequencing and examined in a cosegregation study with disease status. No evidence of cosegregation with the MODY diabetes subtype was found in the three families (data not shown). A fourth intronic variant (IVS7–95G>A) was identified only in a proband from a family with early-onset type 2 diabetes and was investigated in a similar manner; however, no evidence of cosegregation with disease status or age of disease onset was observed in this family (data not shown). Furthermore, one MODY-X family that was examined in the mentioned genome scan (13) and contributed to the chromosome 6 linkage peak was also included in the present mutation analysis. No variants were observed in this family.

The observation that homozygous $Ncb5or^{-/-}$ knock-out mice present with an early-onset diabetic phenotype with a β-cell defect as a primary observation makes human *NCB5OR* a credible biological candidate gene for a mutation analysis in relation to type 2 diabetes and various diabetes subtypes related to β-cell dysfunction. This is further strengthened by evidence from a MODY-X and an affected sibling pair genome scan showing linkage with a chromosomal area close to the *NCB5OR* locus (13,14).

Alignment of the Gln187Arg and His223Arg variants showed that both the Gln- and His-alleles were conserved between the human and murine NCB5OR proteins. All three amino acids are polar and hydrophilic, but whereas histidine and arginine are both positively charged and an exchange of these is not considered to confer a major change in the protein function as such, the glutamine to arginine substitution results in a change from an amide to an alkaline amino acid. The His223Arg variant is located in the *b5* reductase domain of NCB5OR and the Gln187Arg variant in the hinge region connecting the cytochrome *b5* and *b5* reductase domains. The Gln187Arg substitution was genotyped in 1,246 participants; however, no additional carriers were identified. The patient for whom the variant was

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originally identified was a woman diagnosed with glucose intolerance during her second pregnancy. In pregnancies complicated by GDM the increased demand for insulin due to impaired tissue insulin sensitivity is not readily met because of reduced pancreatic β-cell function (rev. in 15). Furthermore, a positive family history of diabetes may pose an increased risk of developing GDM in women >30 years of age (16). We genotyped 140 women with GDM in order to investigate the role of the Gln187Arg variant in GDM, but we failed to identify any additional carriers. At the time of her examination, the 49-year-old patient had a BMI of ~30 kg/m^2, her fasting serum lipids were normal, and she was hypertensive. There was no known history of diabetes in her family, and no family members were available for examination. Given her anthropometric variables, her diabetes may be precipitated by obesity-induced insulin resistance. Whether the Gln187Arg variant is involved in decreased β-cell function is not clear from our data.

The His223Arg variant did not show any association with type 2 diabetes or diabetes-related intermediary phenotypes. On the contrary, the IVS5+7del(CT) variant had a lower frequency among type 2 diabetic patients than among glucose-tolerant subjects and correspondingly a slightly lower relative number of mutation carriers. However, none of the quantitative traits investigated in this study were significantly associated with the variant, although we did observe some suggestive tendencies in fasting serum lipid and insulin levels. For both of the variants this may be due to the low allele frequency and the resulting relatively low power to detect quantitative trait differences; larger study populations are clearly needed. Also, there is a need for replication of the initial finding of an association between the IVS5+7del(CT) variant and type 2 diabetes. The observation that the IVS5+7del(CT) variant confers a modestly reduced risk of developing type 2 diabetes is somewhat counterintuitive, and considering the total number of tests performed in the present study a correction for multiple testing is appropriate. Consequently, the differences in allele frequencies and genotype distribution are not statistically significant. Moreover, we were unable to ascertain a specific metabolic phenotype for this observation. Thus, we conclude that the IVS5+7del(CT) variant is not an important contributor in the pathogenesis of type 2 diabetes in the examined population.

A putative relationship between *NCB5OR* and MODY of as yet unknown genetic cause was investigated by analyzing 63 MODY-X patients for mutations and genotyping all promising variants in the patients' family members. However, there was no evidence in the present study to suggest that variation in *NCB5OR* is involved in MODY or early-onset type 2 diabetes. The assessment of *NCB5OR* as a positional candidate gene for MODY was also done by including one MODY-X family in the mutation analysis, which contributed to the previously described chromosome 6 linkage peak. Likewise, this part of the study did not point to any role of *NCB5OR* in the etiology of MODY. A limitation to this conclusion is, however, that variation in regulatory sequences in the *NCB5OR* locus may exist and possibly have an impact on the expression of the protein, thereby influencing diabetes-related traits.

Even though the $Ncb5or^{-/-}$ mice presented with a very convincing nonautoimmune diabetic phenotype, the lack of an association between the identified *NCB5OR* variants and type 2 diabetes and/or quantitative phenotypic traits could be explained by the fact that the heterozygote $Ncb5or^{+/-}$ mice had NGT. On the contrary, for the *Hnf1α* knock-out mouse it was also observed that the heterozygote $Hnf1\alpha^{+/-}$ mice appeared to have NGT (17), while heterozygote mutations in human *HNF1α* are the cause of MODY3 (2), and a frequent Ala98Val polymorphism in this gene has been suggested to associate with intermediary prediabetic phenotypes such as reduced post–oral glucose tolerance test (OGTT) serum insulin and C-peptide release (18,19).

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In summary, we discovered a total of 22 novel nucleotide variants in *NCB5OR*, and in epidemiological studies we invstigated a selection of these variants along with two additional SNPs identified in silico. The IVS5+7del(CT) variant showed significant association with type 2 diabetes in a case-control study, but we failed to relate this finding with prediabetic and/or obesity-related quantitative traits. Further studies of this variant are needed and may involve functional in vitro studies and genotyping of the variant in even larger study samples in order to explore its role in NCB5OR function and expression.

RESEARCH DESIGN AND METHODS

Mutation analysis was performed in three patient groups. The first group consisted of 61 late-onset type 2 diabetic patients (36 men and 25 women) recruited from the outpatient clinic at Steno Diabetes Center. The mean age of the patients was 62 ± 11 years (mean ± SD), age at diagnosis 54 ± 10 years, BMI 29.9 ± 4.8 kg/m^2, and HbA$_{1c}$ 8.1 ± 1.7%. The patients were treated with diet alone (27%), oral hypoglycemic agents (OHAs) (58%), or insulin alone or in combination with OHAs (15%). The second group, which was also recruited from Steno Diabetes Center, consisted of 59 type 2 diabetic patients with age of onset before 40 years (38 men and 21 women) including 6 women with an earlier diagnosis of GDM. The mean age of the patients was 47 ± 10 years, mean age at diagnosis 37 years (range 21–40), BMI 29.6 ± 6.0 kg/m^2, and HbA$_{1c}$ 8.3 ± 1.7%. The patients were treated with diet alone (25%), OHAs (57%), or insulin alone or in combination with OHAs (18%). The third group involved 63 MODY-X patients (32 men and 31 women) recruited from pediatric and endocrinology departments in Denmark. The mean age of the patients was 34 ± 14 years, mean age at diagnosis 17 years (range 9–25), BMI 25.0 ± 4.6 kg/m^2, and HbA$_{1c}$ 7.5 ± 1.8%. The patients were treated with diet alone (33%), OHAs (6%), or insulin alone or in combination with OHAs (61%). The patients were excluded for mutations known to be present in the MODY genes *TCF1*, *HNF4A*, and *GCK*. In these probands diabetes was present in three consecutive generations or in two generations if cousins of the patients were also affected. The case-control study was performed in unrelated type 2 diabetic patients recruited from the out-patient clinic at Steno Diabetes Center and in unrelated NGT subjects sampled at random during 1994 through 1997 at Steno Diabetes Center and Research Centre for Prevention and Health (20). In the group of type 2 diabetic patients (n = 717, 448 men and 269 women) the mean age was 59 ± 10 years, age at diagnosis 53 ± 10 years, BMI 29.1 ± 5.1 kg/m^2, and HbA$_{1c}$ 8.1 ± 1.7%. The patients were treated with diet (37%), OHAs (53%), or insulin alone or in combination with OHAs (10%). In the group of glucose-tolerant subjects (n = 529, 248 men and 281 women) the mean age was 57 ± 10 years and BMI 25.8 ± 3.7 kg/m^2. The group of 140 women with previous GDM was randomly selected from a population of 376 women with GDM diagnosed during 1978 through 1996 at the Department of Diabetes and Pregnancy, Rigshospitalet, Copenhagen University Hospital (21). The mean age at diagnosis of GDM was 32 ± 5 years, and during pregnancy the patients were treated with diet (94%) or insulin (5%). Diabetes was diagnosed according to 1999 World Health Organization criteria (22). All control subjects underwent a standard 75-g OGTT. All participants were Danish Caucasians by self-report. Informed written consent was obtained from all subjects before participation. The study was approved by the Ethical Committee of Copenhagen and was in accordance with the principles of the Declaration of Helsinki II.

Biochemical assays

Blood samples for measurement of serum insulin and plasma glucose levels were drawn after a 12-h overnight fast. The plasma glucose, serum specific insulin, and serum lipid levels (triglyceride and cholesterol) and HbA$_{1c}$ were measured using Steno Diabetes Center routine methods.

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Mutation detection

The 16 exons of *NCB5OR* including intron-exon boundaries (in total 9,007 bp) was divided into 15 segments (552–719 nucleotides) and amplified using PCR in a total volume of 25 μl with primer sequences, $MgCl_2$ concentration, and annealing temperatures as listed in Table 3. All primers were HPLC purified. Equal amounts of known wild-type PCR and sample PCR products were mixed in order to identify homozygous variants. The mixed PCR products were denatured at 95°C for 5 min, renatured at room temperature for 30 min, and analyzed by dHPLC using the Wave DNA Fragment Analysis System with DNASep Technology (Transgenomic, San Jose, CA). WaveMaker 4.1 was applied to analyze the segments for optimal melting profile. The temperatures are listed in Table 3. Aberrantly migrating samples were bidirectionally sequenced using fluorescent chemistry (MWG-Biotech, Ebersberg, Germany).

Genotyping

The His223Arg variant was genotyped by restriction fragment–length polymorphism (RFLP)-PCR (2 mmol/l $MgCl_2$, T_{anneal} 58°C) with forward primer 5′-ACG TTT TGT CTT GAG GGA TTT-3′ and reverse primer 5′-CAT CTT TGT TTT GCA ATG TGA-3′ followed by digestion with *Nla*III. The Gln187Arg variant was genotyped by restriction site generating (RG) PCR-RFLP (2 mmol/l $MgCl_2$, T_{anneal} 55°C) with forward RG primer 5′-GTC ACC ATT GCC ATA TAT ACT AAC-3′ and reverse RG primer 5′-CTG AGT TCC AAA TGC CGG-3′ (mismatched nucleotides are underlined) followed by digestion with *Hpa*II. The IVS5+7(CT)del variant was genotyped using RG PCR-RFLP (1.5 mmol/l $MgCl_2$, T_{anneal} 50°C) with forward RG primer 5′-GAA AGT CTT AAA TGG GAC GT-3′ and reverse primer 5′-GGA GGT TAC AGT GAG CTG AG-3′ followed by digestion with *Aat*II. This assay does not contain an internal restriction site for control of digestion; however, all heterozygous carriers of the deletion were assayed twice for verification of their genotype, and no discrepancies were detected. No homozygous carriers of the deletion were identified. The rs1408932 A/G variant was genotyped using RG PCR-RFLP (2 mmol/l $MgCl_2$, T_{anneal} 58°C) with forward primer 5′-TGA GCT AGT TGA AAA TTT GAG CA-3′ and reverse RG primer 5′-AAG TTG AGA TCA TGC AGC GAT-3′ followed by digestion with *Mbo*I. The rs10080628 variant was genotyped using RG PCR-RFLP (2 mmol/l $MgCl_2$, T_{anneal} 55°C) with forward RG primer 5′-GGC AAC ATG TTT ACC TCA AGG T-3′ and reverse primer 5′-ACA CGT TGC AGT CAC AAA CC-3′ followed by digestion with *Rsa*I. All restriction enzyme digests were separated on 4% agarose gels and visualized by ethidium bromide staining.

Statistical analysis

Fisher's exact test was applied to examine differences in allele frequencies and genotype distributions between diabetic and nondiabetic subjects. A general linear model was used to test variables (or transformed variables) for differences between genotype groups in the sample of unrelated NGT subjects. Genotype and sex were considered fixed factors and age and BMI as covariates. All phenotype analyses were performed using Statistical Package for Social Science (SPSS, Chicago, IL) version 11.5. A P value <0.05 was considered significant. The area under the curve was calculated using the trapezoidal method in 30-min intervals. The insulinogenic index was calculated as fasting serum insulin (in picomoles per liter) subtracted from 30-min post-OGTT serum insulin (in picomoles per liter) and divided by 30-min post-OGTT plasma glucose (in millimoles per liter). The homeostasis model of assessment for insulin resistance was calculated as fasting plasma glucose (in millimoles per liter) multiplied by fasting serum insulin (in picomoles per liter) divided by 22.5. Linkage disequilibrium was estimated as R^2, where $R^2 = 1$ in the case of complete linkage and $R^2 = 0$ in the case of no linkage. R^2 was calculated as described at www.ekstroem.com.

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In silico data analysis

The human *NCB5OR* nucleotide sequence was retrieved at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) with accession numbers BC025380, AL139232, and AL034347. Intron-exon boundary structure was defined at NCBI (Evidence Viewer) and confirmed at Ensembl (www.ensembl.org). A search for additional SNPs was performed using LocusLink at NCBI (www.ncbi.nlm.nih.gov/LocusLink). Protein translations were performed using Baylor College of Medicine Search Launcher (searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) and confirmed with the protein as it was described previously (12). Protein and nucleotide sequence alignments of the human and murine (NM_024195 and BC025438) protein sequences were performed using ClustalW version 1.82 (www.ebi.ac.uk/clustalw/index.html). Primers for PCRs were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Restriction sites for endonucleases were obtained from Webcutter version 2.0 (www.firstmarket.com/cutter/cut2.html). A genetic map relating the chromosomal localization of human *NCB5OR* with the chromosome 6 MODY-X linkage peak was obtained from the Center for Medical Genetics (www.marshfieldclinic.org/research/genetics/) using markers D6S1017 at 63.28 cM, D6S460 at 89.83 cM, and D6S1634 at 92.25 cM (Kosambi cM).

Acknowledgments

This study was supported by the Danish Medical Research Council, the Danish Diabetes Association, the Danish Heart Foundation, the Velux Foundation, and the European Economic Community (BMH4-CT98-3084 and QLRT-CT-1999-00 546).

The authors thank Professor P.D. Meyts for valuable supervision of G. Andersen's PhD project.

Glossary

dHPLC	denaturing high-performance liquid chromatography
GCK	glucokinase
GDM	gestational diabetes mellitus
HNF	hepatocyte nuclear factor
MAF	minor allele frequency
MODY	maturity-onset diabetes of the young
NCBI	National Center for Biotechnology Information
NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
OHA	oral hypoglycemic agent
RFLP	restriction fragment–length polymorphism
RG	restriction site generating
SNP	single nucleotide polymorphism

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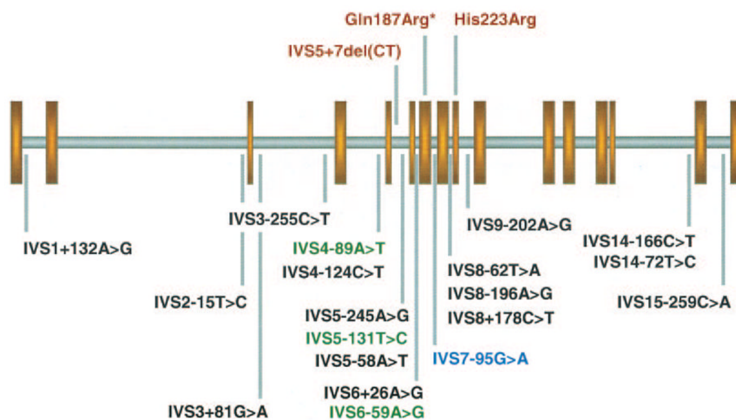

FIG. 1.

Identified variants in *NCB5OR*. A total of 22 variants were observed in the mutation analysis. Three variants (in red) were chosen for genotyping in a case-control study and a genotype-quantitative trait study due to their potential impact on the NCB5OR protein and/ or mRNA expression. Variants in green were each found in only one subject who was the proband of a MODY-X family and were thus genotyped in the remaining family and examined in a family cosegregation study. The variant in blue was observed in only one patient from a family with early onset of type 2 diabetes and was also investigated in a family cosegregation study. *Variant also genotyped in 140 women with GDM.

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TABLE 1

Genotype distribution and allele frequencies for the *NCB5OR* His223Arg and IVS5+7del(CT) variants among type 2 diabetic patients and glucose-tolerant subjects

	Type 2 diabetic patients	Glucose-tolerant control subjects	P_{GD}	P_{AF}
His223Arg				
His/His	702 (100)	516 (99)		
His/Arg	3 (0)	4 (1)		
Arg/Arg	0 (0)	0 (0)		
MAF	0.2 (0.0–0.5)	0.4 (0.0–0.8)	0.5	0.5
IVS5+7del(CT)				
CT/CT	710 (99)	508 (98)		
CT/−	4 (1)	10 (2)		
−/−	0 (0)	0 (0)		
MAF	0.3 (0.0–0.6)	1.0 (0.4–1.6)	0.03	0.03

Data are *n* (%) and % MAF (95% CI). The *P* values compare genotype distribution (P_{GD}) and allele frequencies (P_{AF}) between diabetic patients and glucose-tolerant subjects and were calculated using Fisher's exact test. All genotype groups obeyed Hardy-Weinberg equilibrium.

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TABLE 2

Anthropometric and metabolic characteristics of 529 glucose-tolerant Danish Caucasians stratified according to *NCB5OR* His223Arg and IVS5+7del(CT) genotypes

	His/His	His/Arg	*P*	CT/CT	CT/−	*P*
N (men/women)	516 (243/273)	4 (1/3)		507 (234/273)	10 (6/4)	
Age (years)	57 ± 10	49 ± 14		57 ± 10	56 ± 9	
BMI (kg/m^2)	25.8 ± 3.7	25.6 ± 3.7	0.9	25.8 ± 3.8	26.7 ± 1.6	0.5
Fasting serum lipids						
Total cholesterol (mmol/l)	5.9 ± 1.1	5.4 ± 1.5	0.5	5.9 ± 1.1	5.5 ± 1.0	0.3
HDL cholesterol (mmol/l)	1.5 ± 0.4	1.7 ± 0.5	0.4	1.5 ± 0.4	1.4 ± 0.4	0.6
Triglyceride (mmol/l)	1.3 ± 0.7	1.3 ± 0.4	0.6	1.3 ± 0.7	1.1 ± 0.3	0.4
Plasma glucose						
Fasting (mmol/l)	5.1 ± 0.4	4.9 ± 0.3	0.7	5.1 ± 0.4	5.0 ± 0.3	0.4
30-min post-OGTT (mmol/l)	7.7 ± 1.4	7.4 ± 1.3	0.9	7.7 ± 1.4	7.6 ± 1.1	0.6
60-min post-OGTT (mmol/l)	7.5 ± 2.0	8.2 ± 2.1	0.2	7.5 ± 2.0	8.2 ± 1.7	0.3
120-min post-OGTT (mmol/l)	5.5 ± 1.2	6.1 ± 1.1	0.2	5.5 ± 1.2	5.5 ± 0.9	1.0
Post-OGTT AUC (min · mmol/l)	196 ± 119	256 ± 139	0.2	196 ± 120	232 ± 115	0.4
Serum insulin						
Fasting (pmol/l)	39 ± 20	38 ± 18	0.8	49 ± 20	39 ± 24	0.1
30-min post-OGTT (pmol/l)	259 ± 149	219 ± 109	0.5	258 ± 149	253 ± 165	0.4
60-min post-OGTT (pmol/l)	310 ± 198	390 ± 208	0.3	309 ± 198	318 ± 190	0.7
120-min post-OGTT (pmol/l)	183 ± 123	246 ± 122	0.2	182 ± 124	162 ± 108	0.3
Post-OGTT AUC (min · pmol/l)	23,018 ± 13,281	27,488 ± 14,212	0.4	22,953 ± 13,277	22,662 ± 12,818	0.5
Insulinogenic index	29 ± 18	26 ± 14	0.5	29 ± 18	29 ± 20	0.5
HOMA-IR (mmol/l · pmol/l)	9.0 ± 4.8	8.2 ± 3.8	0.8	9.0 ± 4.8	8.6 ± 5.2	0.1

Data are means ± SD. Values of insulin or derived from insulin variables were logarithmically transformed before statistical analysis. Calculated *P* values were adjusted for age, sex, and BMI. AUC, area under the curve; HOMA-IR, homeostasis model assessment for insulin resistance.

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TABLE 3

Primer sequences, PCR conditions, and dHPLC settings for mutation analysis of *NCB5OR*

Region	Sequence 5′→3′	Product size (bp)	PCR $MgCl_2$ (mmol/l)	dHPLC temperature (°C)
Exon 1	AGCACCAAGGCTAAGGAACA TAGGGGAAAGGGAGGAGGTA	573	2	64.5/66.3
Exon 2	TGAGCATCCTGGATTTTGGT TCAAGCACAAGGCATATTTTT	560	3	53.4/54.9
Exon 3	TATCTGCCAGGACCCATGA AAGGAGGCAGCGAACACTTA	560	2	52.7/56.3
Exon 4	GCATCAGAGCAAATGTCAAAA TGAAGGAAAACCTCTGGAGTG	552	2	55.0/56.7/58.4
Exon 5	AACACGGATGAAACTTGACG AGGCAGGAGAATTGCTTGAA	560	2	53.1/53.7
Exon 6	TGGACAAAAAGCCAGACTAAA TGGAACAGCTCATTCTCCAA	581	3	52.1/53.3/54.8
Exon 7	TGTTGTTTTGGTTTTGCTCTG ACTGCCTGAGTTCCAAATGC	600	3	52.0/52.7
Exon 8	TGAAGGAGAAGAACTGGGTTG TTGTAGGTGTCAGGCACGAG	553	3	53.1/54.0
Exon 9	CCTGCTCCTCTCTTGGTTTG AAACCCCCATGACACACATT	582	2	53.5/54.2
Exon 10	TGTTTGAAAACCAGCAACACA GCACAAGTGGGCTTCTTCAT	649	3	52.8/55.6
Exon 11	GGCATTTTAGTGAAATTGAAGC CATGGAGGACTATTTGTGCAG	574	2	53.4/55.2/57.3
Exon 12	TGAAAATTGTTTCGGCACAC AGAACTTAGCGGCTGACTGG	623	2	52.0/54.7/55.6
Exons 13 and 14	TAGTTTCCCCGCTGGGTTT GCTTCCTGGACAATCAAAGC	719	2	53.6/54.5
Exon 15	CAAACAATCCTCCCACCTTG TAATCCTGGCCTCCAACTCA	658	2	52.3/56.1
Exon 16	GGGTCTGGGGGATCTTACA TGGCAAGGAAATTTGTGATTC	663	2	53.2/54.2

The annealing temperature used in the PCR was 58°C for all segments except exon 15 (60°C). The dHPLC linear elution buffer gradient was 62% for all segments except exons 10 and 13–16, where it was 63%.