Diabetologia (1997) 40: 980–983

Diabetologia

Studies of the genetic variability of the coding region of the hepatocyte nuclear factor-4α in Caucasians with maturity onset NIDDM

A. M. Møller[1], S. A. Urhammer[1], L. T. Dalgaard[1], R. Reneland[2], L. Berglund[2], T. Hansen[1], J. O. Clausen[3], H. Lithell[2], O. Pedersen[1]

[1] Steno Diabetes Center and Hagedorn Research Institute, Denmark
[2] Department of Geriatrics, Uppsala University, Uppsala, Sweden
[3] Center of Preventive Medicine, Glostrup University Hospital, Copenhagen, Denmark

Summary Mutations in the hepatocyte nuclear factor-4α (HNF-4α) gene cause the type 1 form of maturity onset diabetes of the young (MODY1). To address the question of whether genetic variability of HNF-4α is associated with late onset non-insulin-dependent diabetes mellitus (NIDDM) we have sequenced the coding region and intron/exon boundaries of the gene in 36 randomly recruited Danish NIDDM patients. Two nucleotide substitutions that changed the sequence of HNF-4α were identified: Thr/Ile130, which has been reported previously and a novel Val/Met255. The Val/Met 255 mutation was found in 4 of 477 Danish NIDDM patients and in none of 217 glucose tolerant control subjects; thus it cannot be excluded that this mutation may have an impact on NIDDM susceptibility. Among 509 NIDDM patients the allelic frequency of the Thr/Ile130 variant was 4.7 % (95 % confidence interval: 3.4–6.0 %) compared to 1.9 % (0.7–3.1 %) among 239 control subjects ($p = 0.008$). However, in a population sample of 942 Swedish men with an average age of 70 years the allelic frequency of the variant was similar in 246 men with either impaired glucose tolerance (5.6 % [2.6–8.6 %]) or NIDDM (5.4 % [2.7–8.1 %]) as compared to 666 glucose tolerant men (5.1 % [3.9–6.3 %]). Also in a population sample of 369 young healthy Danes the prevalence of the codon 130 variant (4.7 % [3.2–6.2 %]) was similar to what was found in Swedish Caucasians. Thus, the allelic frequency of the Thr/Ile130 variant among the control subjects in the Danish case-control study deviates from the prevalence in the two other studies which is why we consider the significant association between the codon 130 variant and NIDDM an incidental finding. In glucose tolerant subjects the codon 130 variant in its heterozygous form had no major effect on glucose-induced insulin and C-peptide release although a tendency to a lower insulin secretion during an oral glucose tolerance test was seen in middle-aged subjects. In conclusion, variability in the coding region of the HNF-4α gene is not a common cause of NIDDM among whites of Danish ancestry. However, a Val/Met255 mutation was found exclusively in NIDDM patients (0.8 % of cases) and functional as well as family segregation studies are needed to determine whether this HNF-4α variant is a NIDDM causing mutation. [Diabetologia (1997) 40: 980–983]

Keywords Hepatocyte nuclear factor-4α, non-insulin-dependent diabetes mellitus, insulin response, mutations, transcription factors.

Received: 17 April 1997 and in revised form: 28 May 1997

Corresponding author: A. M. Møller, MS. Sci., Steno Diabetes Center, Niels Steensens Vej 2, DK-2820 Gentofte, Copenhagen, Denmark

Abbreviations: HNF-4α, Hepatocyte nuclear factor-4α; MODY, maturity onset diabetes of the young; NIDDM, non-insulin dependent diabetes mellitus; OGTT, oral glucose tolerance test; IVGTT, intravenous glucose tolerance test.

AM Møller and SA Urhammer contributed equally to this study

Hepatocyte nuclear factor (HNF)-4α is a member of the steroid/thyroid hormone receptor superfamily and is expressed in the liver, kidney, small intestine and pancreatic islets [1, 2]. It activates a wide variety of essential genes, including those involved in cholesterol, fatty acid and glucose metabolism and in liver differentiation [3]. Mutations in the HNF-4α gene are the cause of the type 1 form of maturity onset diabetes of the young (MODY1) [4]. Targeted disruption of the HNF-4α gene in mice results in embryonic

Table 1. Nucleotide sequences of DNA primers used for PCR amplification of the HNF-4α gene

Exon	Sense primer (5' → 3')	Antisense primer (5' → 3')	Segment size	$T_{annealing}/C_{MgCl}$
1	tgtaaaacgacggccagtgggcactgggaggaggcagt	caggaaacagctatgacccttggcaacacctgtgctggc	405 bp	65 °C/1.5 mmol/l
1b	tgtaaaacgacggccagttcatatcagcaacatgtccg	caggaaacagctatgaccgggctcttccctccagga	210 bp	60 °C/2 mmol/l
2	tgtaaaacgacggccagtcttcctgaagcctcactcc	caggaaacagctatgacccccaagtgtgcccatttcc	352 bp	60 °C/2 mmol/l
3	tgtaaaacgacggccagtgttgtgtcttctccatcca	caggaaacagctatgaccgcaggtggggcagtggtg	215 bp	55 °C/1.5 mmol/l
4	tgtaaaacgacggccagttctccctcctcacctctctg	caggaaacagctatgacccctctgtagtgtgggggga	226 bp	65 °C/1.5 mmol/l
5	tgtaaaacgacggccagtatctccagcattttcttccc	caggaaacagctatgacccactgcccactactgccc	267 bp	55 °C/2 mmol/l
6	tgtaaaacgacggccagtagggtacagatggcaaacac	caggaaacagctatgaccaccctccctggagccctg	204 bp	65 °C/2 mmol/l
7	tgtaaaacgacggccagttgacttcccatcctccctcc	caggaaacagctatgaccggagagagagtcagggatgg	268 bp	60 °C/2 mmol/l
8	tgtaaaacgacggccagtagctggaccctgctgccc	caggaaacagctatgacccactccaaccccgcccct	354 bp	60 °C/2 mmol/l
9	tgtaaaacgacggccagtgcatcccagactctccatcc	caggaaacagctatgaccttgcaaggtaaaatcccagag	262 bp	60 °C/1.5 mmol/l
10	tgtaaaacgacggccagtagcccctgtctgtctgtttg	caggaaacagctatgaccgggactggtcctggcatcac	316 bp	60 °C/2 mmol/l

"T_{anneal}" denotes annealing temperature in the polymerase chain reaction (PCR). "C_{MgCl2}" denotes the $MgCl_2$ concentration in the PCR

lethality indicating the key position of this transcription factor in cell development and differentiation [5]. HNF-4α is a major activator of HNF-1α, which is required for the tissue-specific expression of genes in tissues such as liver, kidney and pancreas. MODY3 is caused by mutations in the HNF-1α gene [6]. Recently, markers flanking the HNF-4α gene on chromosome 20q have been linked to late-onset non-insulin-dependent diabetes mellitus (NIDDM) in Caucasian subjects [7, 8] implying that defects in this gene might contribute to the development of NIDDM. In this study we have screened the HNF-4α gene for variants in 36 Danish NIDDM patients and addressed the questions of whether genetic variability of the coding region of the HNF-4α gene might: 1) be associated with an increased prevalence of NIDDM; and 2) affect pancreatic beta-cell function.

Subjects and methods

Subjects. In the primary mutational screening direct sequencing was performed on genomic DNA from 36 randomly chosen Danish Caucasian NIDDM patients who were diagnosed after the age of 40 years. The subsequent association studies were done in 509 unrelated Danish Caucasian NIDDM patients recruited from the outpatient clinic at Steno Diabetes Center, Copenhagen, and 240 age-matched, unrelated and glucose tolerant Danish Caucasian control subjects traced randomly in the Danish Central Population Register and living in the same area of Copenhagen as the NIDDM patients. NIDDM was diagnosed by World Health Organization criteria and all control subjects underwent a standard 75-g oral glucose tolerance test (OGTT) with measurements of plasma glucose, serum insulin and C-peptide during the test. For studies of the acute (0–8 min) glucose induced insulin and C-peptide responses during an intravenous glucose tolerance test (IVGTT), 380 subjects were recruited from a population sample of young individuals aged 18–32 years. Details of the IVGTT and physiological characteristics of this population sample have been presented previously [9]. The studies were approved by the Ethical Committee of Copenhagen. Also a Swedish cohort of 1192 men was examined for the HNF-4α variants. All these subjects underwent a standard OGTT from which NIDDM was diagnosed according to National Diabetes Data Group (NDDG) criteria. The aim and the details of this study have been reported previously [10]. This study was approved by the Human Ethics Committee of the Medical Faculty of Uppsala University. Prior to the participation in the study informed consent was obtained from all studied subjects. All the studies were carried out in accordance with the principles of the Declaration of Helsinki.

Biochemical variables. In the Danish association study and in the study of 380 young Danes plasma concentration of glucose was analysed by an automated glucose oxidation method (Granutest; Merck, Darmstadt, Germany). The concentration of specific insulin (excluding des(31,32)- and intact proinsulin) in serum was measured by ELISA and the concentration of serum C-peptide was determined by radioimmunoassay RIA by using Steno Diabetes Center routine methods. In the Swedish study plasma glucose was measured by the glucose dehydrogenase method (gluc-DH; Merck, Darmstadt, Germany).

Identification of mutations in the HNF-4α gene. The 11 exons and flanking introns were amplified using PCR (model 9600; Perkin Elmer/Cetus, Foster City, Calif., USA) and specific primers (Table 1). PCR conditions were: denaturation at 94 °C for 9 min followed by 35 cycles of denaturation for 30 s, annealing at T_{anneal} (Table 1) for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 9 min. PCR products were purified using Microcon 100 microconcentrators and sequenced directly using ABI PRISM Dye Primer Cycle Sequencing Kit with Amplitaq DNA Polymerase FS and ABI prism 377 (Perkin Elmer).

Double stranded sequencing was performed in cases of difficulties in the interpretation of the nucleotide sequencing of one strand. Furthermore, all nucleotide variants were identified on both strands.

Screening for amino acid polymorphisms in the HNF-4α gene. PCR amplification of the DNA segment containing codon 130 was carried out using specific primers [4]. Restriction fragment length polymorphisms were detected after digestion with *BstB* I. The fragments were resolved on a 3 % agarose gel and visualized by staining with ethidium bromide. The Val/Met255 variant was detected by restriction fragment length polymorphism-generating polymerase chain reaction using sense primer 5'-ccggagctggcggagatgacccg-3' and antisense primer 5'-caggaaacagctatgaccggagagagagtcagggatgg-3' (annealing at 65 °C) and digestion with *Ava* I. The fragments were resolved on a 4 % agarose gel and visualized by staining with ethidium bromide.

Statistical analysis. Chi-square analysis and Fishers exact test when appropriate were applied to test for significance of

Table 2. Clinical and biochemical data of 239 middle-aged healthy Danish Caucasians when classified in accordance to their genotype of the codon 130 amino acid polymorphism of the hepatocyte nuclear factor-4α gene

	Genotype		
	TT-130	TI-130	*p* value
n	230	9	
Sex (men/women)	117/113	4/5	
Age (year)	52 (20)	52 (19)	0.50
BMI (kg/m^2)	24.9 (4.6)	23.5 (7.4)	0.38
Fasting plasma glucose (mmol/l)	5.1 (0.8)	5.0 (0.6)	0.25
Fasting serum C-peptide (pmol/l)	525 (205)	485 (183)	0.77
Fasting serum insulin (pmol/l)	37 (25)	27 (27)	0.50
Plasma glucose 30 min[a] (mmol/l)	7.6 (1.8)	7.9 (2.8)	0.52
Serum C-peptide 30 min[a] (pmol/l)	1590 (680)	1360 (673)	0.50
Serum insulin 30 min[a] (pmol/l)	236 (151)	171 (73)	0.51
AUC-glucose (min × mmol/l)	170 (164)	272 (300)	0.64
AUC-insulin (min × pmol/l)	19710 (13748)	16043 (10920)	0.15
AUC-C-peptide (min × pmol/l)	137025 (69675)	114150 (35194)	0.28
Ratio (AUC-insulin/AUC-glucose)	31 (19)	24 (6)	0.03
Ratio (AUC-C-peptide/AUC-glucose)	263 (92)	228 (88)	0.20

Values are medians (interquartile range).
The *p* value compares subjects heterozygous (TI-130) for the codon 130 polymorphism in the hepatocyte nuclear factor-4α gene with homozygous wild type carriers (TT-130). AUC is incremental area under the curve (0–120 min) of plasma glucose, serum C-peptide or serum insulin during the OGTT.
[a] Sampled at 30 min during the OGTT

differences in allele frequencies. Differences in continuous variables between groups of subjects were tested with Student's *t*-test when the distributions of the variables or the logarithmic values of the variable were normal and when the variances of the variables were equal in the compared groups. Otherwise the Mann-Whitney test was used. Data are medians (interquartile ranges). To control for possible confounders on AUC-insulin and AUC-C-peptide multiple linear regression analyses were performed. Sex, BMI and AUC-glucose were entered as explanatory variables and AUC-insulin and AUC-C-peptide, respectively, were response variables. If necessary the variables entered in the analysis were logarithmically transformed. A *p* value less than 0.05 (two-tailed) was considered significant. Statistical Package of Social Science (SPSS) for Windows, version 7.0 was used for statistical analysis.

Results

Direct sequencing of the HNF-4α gene in 36 NIDDM patients revealed 5 nucleotide substitutions, of which 3 were located in exons and 2 in introns. In the first intron 33 bp downstream of exon 1 a G → A substitution was identified and 5 bp upstream of exon 2 a C → T substitution was found. In exon 2 at codon 58 a silent Ala(GCC) → Ala(GCT) nucleotide change was identified. A novel G → A substitution at codon 255 resulting in a Val(GTG) → Met(ATG) replacement was detected in 4 out of 477 (of 509) subjects with NIDDM and in none of 217 (of 240) non-diabetic control subjects (NS). The 4 patients were heterozygous for this sequence variant. Their ages at onset, BMI, fasting serum insulin and C-peptide level, or mode of treatment did not differ from the wild type carriers with NIDDM (data not shown). Family members of the Val/Met255 carriers were not available for examination.

At codon 130 a C → T substitution resulting in a Thr(ACT) → Ile(ATT) replacement was identified. The allelic frequency of this polymorphism among 509 (of 509) NIDDM patients was 4.7 % (95 % confidence interval: 3.4–6.0 %) and 1.9 % (0.7–3.1 %) among 239 (of 240) control subjects ($\chi^2 = 7.1$, $p = 0.008$). Among 369 (of 380) young healthy subjects the allelic frequency of the Thr/Ile130 variant was 4.7 % (3.2–6.2 %). All the observed genotype frequencies were in Hardy-Weinberg equilibrium. Among the 239 glucose tolerant control subjects 9 were heterozygous for the Thr/Ile130 polymorphism. Although, the TI-130 carriers had normal levels of serum C-peptide and serum insulin during the OGTT compared to subjects carrying the TT-130 genotype (data not shown), the ratio AUC-insulin/AUC-glucose (ratio between the area under the insulin curve and the area under the glucose curve) was reduced by 23 % in TI-130 carriers compared to TT-130 carriers ($p = 0.03$) (Table 2). After Bonferroni correction for multiple testing the statistic significance disappeared. When adjusting for gender, BMI and AUC-glucose in a multiple regression analysis the AUC-insulin still tended, however, to be lower ($p = 0.06$) among heterozygous carriers compared to wild type carriers.

In the genotype-phenotype study of 369 young subjects 33 were heterozygous and 1 subject was homozygous for the Thr/Ile130 polymorphism. There were no differences in the acute (0–8 min) serum insulin response (median (interquartile range): 1867 (1444) vs 1965 (1646) min × pmol/l) or serum C-peptide response (6275 (3515) vs 6345 (4400) min × pmol/l) during an IVGTT between heterozygous carriers and wild type carriers of the variant. Furthermore, there were no significant differences between

genotypes in age, BMI, fasting values of plasma glucose, serum insulin and serum C-peptide, $AUC_{(0–8\ min)}$-insulin/$AUC_{(0–8\ min)}$-glucose and $AUC_{(0–8\ min)}$-C-peptide/$AUC_{(0–8\ min)}$-glucose (data not shown). The one II-130 homozygote had low levels of acute serum insulin (749 min × pmol/l) and C-peptide (3890 min × pmol/l) responses as compared to TT-130 wild type carriers.

In the cohort from Uppsala comprising 942 (of 1192) men with an average age of 70 years the allelic frequency of the codon 130 variant was 5.1% (3.9–6.3%) among 666 glucose tolerant men as compared to 5.6% (2.6–8.6%) among 116 men with IGT and 5.4% (2.7–8.1%) among 130 NIDDM subjects. The genotype frequencies were in Hardy-Weinberg equilibrium. The Val/Met255 mutation was not found in 894 (of 1192) Swedish men.

Discussion

The analysis of the sequence of the HNF-4α gene in NIDDM patients revealed five nucleotide substitutions, including two substitutions that altered the amino acid sequence of the HNF-4α protein. The Val/Met255 replacement is a novel variant that was found in 4 diabetic subjects (0.8%) and in none of the control subjects. This substitution changes an amino acid that is conserved among several species, i.e. human, rat and mouse and is located in the putative ligand binding domain of the HNF-4α protein [1], which is important for the activity of the protein. Although none of the Val/Met255 carriers have overt hepatic, renal or gastrointestinal dysfunction the variant might have an effect on the pancreatic beta-cell function contributing to the development of NIDDM. Thus, the potential impact of this amino acid replacement on HNF-4α function needs to be examined directly. Also segregation studies in families may provide information of the potential diabetogenic influence of this variant.

The Thr/Ile130 polymorphism, which changes an amino acid that is conserved in xenopus, mouse, rat and human, has previously been reported in a group of 55 unrelated non-diabetic non-Hispanic white subjects with an allelic frequency of 5% [4]. In the Danish NIDDM patients the allelic frequency of the codon 130 variant was similar to the prevalence reported among non-Hispanic whites whereas the matched glucose tolerant Danes had an allelic frequency which was less than half. Although the Danish control subjects were carefully sampled and matched we consider their low frequency of the codon 130 variant an incidental finding. This interpretation of data is based on the fact that the Thr/Ile130 polymorphism had a similar allelic frequency (about 5%) in young Danish Caucasians and in Swedish Caucasians without or with impairment of glucose tolerance.

The Thr/Ile130 polymorphism in its heterozygous form was clearly not associated with altered insulin or C-peptide responses after intravenous glucose in the population sample of subjects aged 18–32 years. A tendency to a lower insulin response following an OGTT was found in middle-aged glucose tolerant subjects who were heterozygous carriers of the variant. However, due to the potential pitfalls of case-control comparisons this finding should be interpreted with caution until it has been subjected to the test of independent replication.

In conclusion; even though we cannot exclude an effect of the Val/Met255 amino acid replacement on NIDDM susceptibility, variability in the coding region of the HNF-4α gene is not a common cause of NIDDM among whites of Danish ancestry.

Acknowledgements. The study was supported by grants from the University of Copenhagen, the Velux Foundation, the Danish Diabetes Association, EEC grant (BMH4-CT-950662) and the Danish Medical Research Council. The authors thank Sandra Urioste, Annemette Forman, Lene Aabo, Helle Fjordvang, Bente Mottlau, Susanne Kjellberg, Lis Ølholm and Marja Lis Halkjar for dedicated and careful technical assistance and Grete Lademann for secretarial support.

References

1. Sladek FM, Zhong W, Lai E, Darnell Jr JE (1990) Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Development 4: 2353–2365
2. Miquerol L, Lopez S, Cartier N, Tulliez M, Raymondjean M, Kahn A (1994) Expression of the L-pyruvate kinase gene and the hepatocyte nuclear factor 4 transcription factor in exocrine and endocrine pancreas. J Biol Chem 269: 8944–8951
3. Jian G, Nepomuceno L, Hopkins K, Sladek FM (1995) Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. Molecular and Cellular Biology 15: 5131–5143
4. Yamagata K, Furota H, Oda N et al. (1996) Mutations in the hepatocyte nuclear factor-4 gene in maturity-onset diabetes of the young (MODY1). Nature 384: 458–460
5. Chen WS, Manova K, Weinstein DC et al. (1994) Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Development 8: 2466–2477
6. Yamagata K, Oda N, Kaisaki PJ et al. (1996) Mutations in the hepatocyte nuclear factor-1 gene in maturity-onset diabetes of the young (MODY3). Nature 384: 455–458
7. Hani EH, Zouali H, Philippi A et al. (1996) Indication for genetic linkage of the phosphoenolpyruvate carboxykinase (PCK1) gene region on chromosome 20q to non-insulin dependent diabetes mellitus. Diab Metab 22: 451–454
8. Ji L, Malecki M, Warram JH, Yang Y, Rich SS, Krolewski AS (1977) New susceptibility locus for NIDDM is localized to human chromosome 20q. Diabetes 46: 876–881
9. Clausen JO, Borch-Johnsen K, Ibsen H et al. (1996) Insulin sensitivity index, acute insulin response, and glucose effectiveness in a population-based sample of 380 young healthy Caucasians. Analysis of the impact of gender, body fat, physical fitness, and lifestyle factors. J Clin Invest 98: 1195–1209
10. Hedstrand H (1975) A study of middle-aged men with particular reference to risk factors for cardiovascular disease. Uppsala J Med Sci 80 [Suppl 19]: 1–61