Short communication

Identification of missense mutations in the hepatocyte nuclear factor-3β gene in Japanese subjects with late-onset Type II diabetes mellitus

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Abstract

Aims/hypothesis. Hepatocyte nuclear factor (HNF)-3β, a transcription factor expressed in pancreatic beta cells, is an upstream regulator of *HNF-1α*/MODY3, *HNF-4α*/MODY1 and *IPF1/MODY5* genes. Our previous screening of MODY subjects showed that mutations in the *HNF-3β* gene are not a common cause of this form of diabetes in the Japanese. We tested the hypothesis that mutations in the *HNF-3β* gene cause late-onset Type II (non-insulin-dependent) diabetes mellitus in this population.
Methods. Genotyping of the polymorphic TCC repeat in the *HNF-3β* gene was done in 112 Japanese subjects with Type II diabetes (age at diagnosis > 35 and family history of Type II diabetes among their second-degree relatives) and 96 Japanese control subjects. Furthermore, we screened 57 Type II diabetic patients for mutations of the *HNF-3β* gene.

Transactivation activity of variant HNF-3β was investigated by transfection assay.
Results. The distribution of alleles of the TCC repeat was similar between diabetic and control groups. Mutation screening identified two missense mutations, A86T and G114E. Neither mutation was observed in 225 control subjects. The transactivation activity of G114E-HNF-3β was similar to that of wild type-HNF-3β. In contrast, the activity of A86T-HNF-3β was statistically significantly reduced to 83–86% of that of wild type.
Conclusions/Interpretation. The A86T mutation in the *HNF-3β* gene might be involved in the development of late-onset Type II diabetes in a small group of Japanese people. [Diabetologia (2000) 43: 1197–1200]

Keywords Keywords. HNF-3β, HNF-1α, mutation, genetics.

Maturity-onset diabetes of the young (MODY) is an autosomal dominant form of early-onset Type II (non-insulin-dependent) diabetes mellitus. Recent genetic studies found that mutations in the genes encoding hepatocyte nuclear factor (HNF)-1α, HNF-1β, HNF-4α and insulin promoter factor-1 (IPF-1)

are causes of MODY3, MODY5, MODY1 and MODY4, respectively [1–3]. Hepatocyte nuclear factor –1α, HNF-1β and HNF-4α are functionally related transcription factors which are important for the pancreatic beta-cell functions. Insulin promoter factor –1 regulates the expression of the insulin and glucokinase/*MODY2* genes. These findings suggest that the transcription factors expressed in beta cells are important. Hepatocyte nuclear factor –3β is a transcription factor containing a winged-helix DNA binding domain and is involved in the regulation of the expression of *HNF-1α*/MODY3, *HNF-4α*/MODY1 and *IPF-1/MODY5* genes [4, 5]. This raises the possibility that mutations in the *HNF-3β* gene cause a decrease in the synthesis of these transcription factors and lead to diabetes. We recently cloned cDNA and the

gene encoding human HNF-3β [6]. Mutation screening in 45 Japanese subjects with MODY showed that mutations in the *HNF-3β* gene are not associated with MODY in the Japanese [6]. Linkage studies for common Type II diabetes susceptible loci in a large sample of affected sib-pairs in Finnish families have, however, identified a linkage peak around the *HNF-3β* gene on chromosome 20 [7]. In this study, we examined whether mutations in the *HNF-3β* gene are involved in the pathogenesis of late-onset Type II diabetes in Japanese subjects.

Subjects and methods

Subjects and screening of the HNF-3β gene. In this study, we studied unrelated Japanese subjects who had been diagnosed with Type II diabetes after 35 years of age and had a family history of Type II diabetes among their second-degree relatives. We tested 112 unrelated Japanese subjects with Type II diabetes and 96 unrelated Japanese control subjects for genotyping using a polymorphic TCC repeat in the intron 1. Polymerase chain reaction was carried out as described previously using a kit of TaKaRa LA*Taq* with GC buffer (TaKa-Ra, Tokyo, Japan) and 6-carboxy fluorescein labelled primers (5'-CTATATCACCAGCCTCCCACGTCAC-3' and 5'-GTTTCTTCTGAGGTTGGCAGTGCCGAGCTG-3') [6]. The PCR products were analysed with ABI Prism 377 DNA Sequencer and ABI Prism GeneScan Analysis Software (version 2.1) (PE Applied Biosystems, Foster City, Calif., USA). GeneScan-400HD ROX (PE Applied Biosystems) was used as a size control.

We did DNA sequencing of the coding regions and flanking introns of the HNF-3β gene [6] in 57 unrelated Japanese Type II diabetic patients. The occurrence of A86T (loss of *Nar*I site) and G114E (gain of *Xho*I site) mutations in 225 control subjects were determined by PCR-restriction fragment length polymorphism (RFLP) and DNA sequencing. Written informed consents were obtained from all of the participants and the study was carried out in accordance with the Declaration of Helsinki as revised in 1996.

Construction of plasmids and reporter gene assay. Human HNF-3β cDNA was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, Calif., USA). We introduced A86T and G114E mutations by in vitro mutagenesis. Three copies of the HNF-3β binding site of the mouse transthyretin (TTR) promoter were inserted upstream of the HSV-TK promoter/luciferase fusion gene (pHNF3β-tk-Luc). Reporter constructs with HNF-1α promoter (HNF-1α-pGL3) and TTR promoter (TTR-pGL3) have been described previously [8]. We transfected HeLa cells (1.5 × 10^5 cells/well) with 100 ng of HNF-3β expression and 500 ng of reporter vectors together with 10 ng of pRL-SV40 (Promega, Madison, Wis., USA) as an internal control using the LIPOFECTAMINE PLUS Reagent (Life Technologies, Rockville, Md., USA). The transactivation activity of wild-type (WT)-HNF-3β, A86T-HNF-3β and G114E-HNF-3β was measured after 48 h using the Dual Luciferase Reporter Assay System (Promega). Renilla luciferase activity was used to normalize transfection efficiencies among experiments. Each experiment was repeated six times.

Statistical analysis. The statistical significance of the differences was assessed using the unpaired-Student's *t* test. We considered *p* less than 0.05 as significant.

Results

Genotyping of the TCC repeat. We observed ten different alleles (Table 1). The distribution of the alleles was similar between diabetic and control groups, indicating that this *HNF-3β* polymorphism is not associated with late-onset Type II diabetes in Japanese subjects.

Identification of variants in HNF-3β gene. Screening of the *HNF-3β* gene for mutations in 57 Japanese Type II diabetic patients with family histories of diabetes identified two missense mutations (A86T (<u>G</u>CC to <u>A</u>CC) and G114E (G<u>G</u>G to G<u>A</u>G)), two silent mutations in the codons for Ala97 (GCC to GC<u>T</u>) and Gly279 (GG<u>A</u> to GG<u>G</u>)) and one in the 3' untranslated region (C/T; + 49 nt after stop codon). The existence of missense mutations was confirmed by the sequencing of the subcloned PCR products. The A86T mutation was identified in two unrelated subjects with Type II diabetes and this mutation alters an amino acid that is located in the transcriptional activation domain (region V) [9]. The other missense mutation G114E is located in the linker region between the transcriptional activation domain and DNA binding domain. Neither mutation was found in 225 Japanese control subjects. To assess whether A86T and G114E are common mutations in Japanese subjects with late-onset Type II diabetes, we studied more diabetic patients. The A86T and G114E mutations were not observed in the 175 and 95 subjects, respectively. Thus, frequencies of A86T and G114E in Japanese subjects with late-onset Type II diabetes were 0.8 % (2/232) and 0.6 % (1/152), respectively. These data suggest that neither mutation is a common cause of late-onset Type II diabetes in this cohort. The frequencies of the three other silent mutations were similar between diabetic and control groups (Table 2).

Clinical profiles of subjects with HNF-3β mutation. We identified the A86T mutation in two unrelated subjects with Type II diabetes. Subject 1 is a 54-year-

Table 1. Allele distribution of the polymorphic TCC repeat in the *HNF-3β* gene

Allele size (bp)	Type II diabetes (*n* = 112)		Control subjects (*n* = 96)	
	Number	Frequency (%)	Number	Frequency (%)
170	1	0.40	0	0.00
193	0	0.00	1	0.52
208	12	5.36	9	4.69
211	5	2.23	3	1.56
214	17	7.59	13	6.77
217	127	56.25	119	61.98
220	1	0.40	4	2.08
223	41	18.33	24	12.50
226	16	7.14	18	9.38
229	4	1.79	1	0.52

Table 2. Mutations/polymorphisms in the *HNF-3β* gene

Region		Nucleotide change	Amino acid change		Allelic frequency	
					Type II diabetes (n = 57)	Control (n = 64)
Exon 3	codon 86	G to A	Ala (GCC) to Thr (ACC)	G/A	0.98/0.02	1.00/0.00
	codon 97	C to T	Ala (GCC) to Ala (GCT)	C/T	0.87/0.13	0.77/0.23
	codon 114	G to A	Gly (GGG) to Glu (GAG)	G/A	0.99/0.01	1.00/0.00
	codon 279	A to G	Gly (GGA) to Gly (GGG)	A/G	0.85/0.15	0.77/0.23
+ 49 nt after stop codon		C to T		C/T	0.89/0.11	0.77/0.23

Allele frequencies in the control subjects are cited from reference [6]

Table 3. Clinical characteristics of the diabetic patients with mutations in the *HNF-3β* gene

	Subject 1	Subject 2	Subject 3
Mutation	A86T	A86T	G114E
Age at diagnosis (years)	44	38	65
Duration of diabetes (years)	10	34	7
Present BMI (kg/m^2)	26.8	17.4	20.2
Present HbA$_{1c}$ (%)	10.8	8.2	7.5
Therapy	Insulin	Insulin	OHA
Complication	PDR, neuropathy and nephropathy	Neuropathy	Retinopathy

OHA, oral hypoglycaemic agent; PDR, proliferative diabetic retinopathy

old woman (BMI = 26.8). She developed Type II diabetes at 44 years of age. She was first treated with an oral hypoglycaemic agent, and with insulin after she was 46 years of age. Her present HbA$_{1c}$ value is 10.8% and she has proliferative diabetic retinopathy, diabetic nephropathy and severe neuropathy. Her present urinary C peptide is 28.2 µg/day (normal range, 45 to 117 µg/day). Her C-peptide response to glucagon is impaired (ΔC-peptide = 0.46 nmol/l per 5 min, normal 1.75 ± 0.32). The proband has six more siblings. Of them three developed late-onset Type II diabetes and have the A86T mutation. Out of six children of the proband's 64-year-old sister, who has the mutation and developed Type II diabetes at 53 years of age, three (present age; 37, 33 and 31 years of age) have inherited the mutant allele. One of the children (33-year-old woman), who also has the mutation, was identified as having glycosuria when she was pregnant at 24 years of age. The other children were not diabetic when interviewed, although glucose tolerance tests have not been carried out on these subjects.

Subject 2 is a 72-year-old man (BMI = 17.4). He developed Type II diabetes at 38 years of age and started insulin treatment 17 years after diagnosis. His present HbA$_{1c}$ value is 8.2% and he has severe neuropathy. His children (38 and 42 years of age) were not diabetic when interviewed.

The proband with the G114E mutation is a 72-year-old woman (BMI = 20.2). She developed Type II diabetes at 65 years of age. She is now being treated with an oral hypoglycaemic agent and her present HbA$_{1c}$ is 7.5%. She has two diabetic sisters, one of whom has the mutant allele.

Clinical data of subjects 1–3 are summarized in Table 3.

Functional analysis of mutant HNF-3β. We studied the functional properties of the mutant HNF-3β proteins. HeLa cells were transfected with WT and mutant HNF-3β constructs together with the pHNF-3β-tk-Luc reporter. We found WT-HNF-3β, A86T-HNF-3β and G114E-HNF-3β activated the reporter gene by 34-fold, 28-fold and 32-fold, respectively (Fig.1). The transactivation activity of A86T-HNF-3β was significantly reduced to 83.4% of that of WT (p = 0.005) but the difference between WT-HNF-3β and G114E-HNF-3β was not significant. The transactivation activity of A86T-HNF-3β was also tested with two other reporters, HNF-1α-pGL3 and TTR-pGL3. The transcription of HNF-1α and TTR reporters by A86T-HNF-3β were significantly decreased compared with WT-HNF-3β: 83.6% (p = 0.00037) and 86.2% (p = 0.0014), respectively (Fig.1).

Discussion

In screening the *HNF-3β* gene in 57 Japanese subjects with late-onset Type II diabetes we found two missense mutations, A86T and G114E, and neither mutation was observed in 225 control subjects. The glycine at codon 114 is not conserved in *Xenopus* and Zebrafish [6]. The transactivation activity of G114E-HNF-3β was similar to that of WT-HNF-3β. These data suggest that the G114E mutation is not a diabetes-associated mutation. In contrast, alanine at codon 86 is conserved in human, mouse, rat, *Xenopus* and Zebrafish [6] and the A86T mutation was present in all (4/4) of the diabetic members of subject 1's family. The transactivation activity of A86T-HNF-3β was statistically significantly reduced compared with WT-HNF-3β. The physiological importance of such a modest reduction for the ability to develop Type II
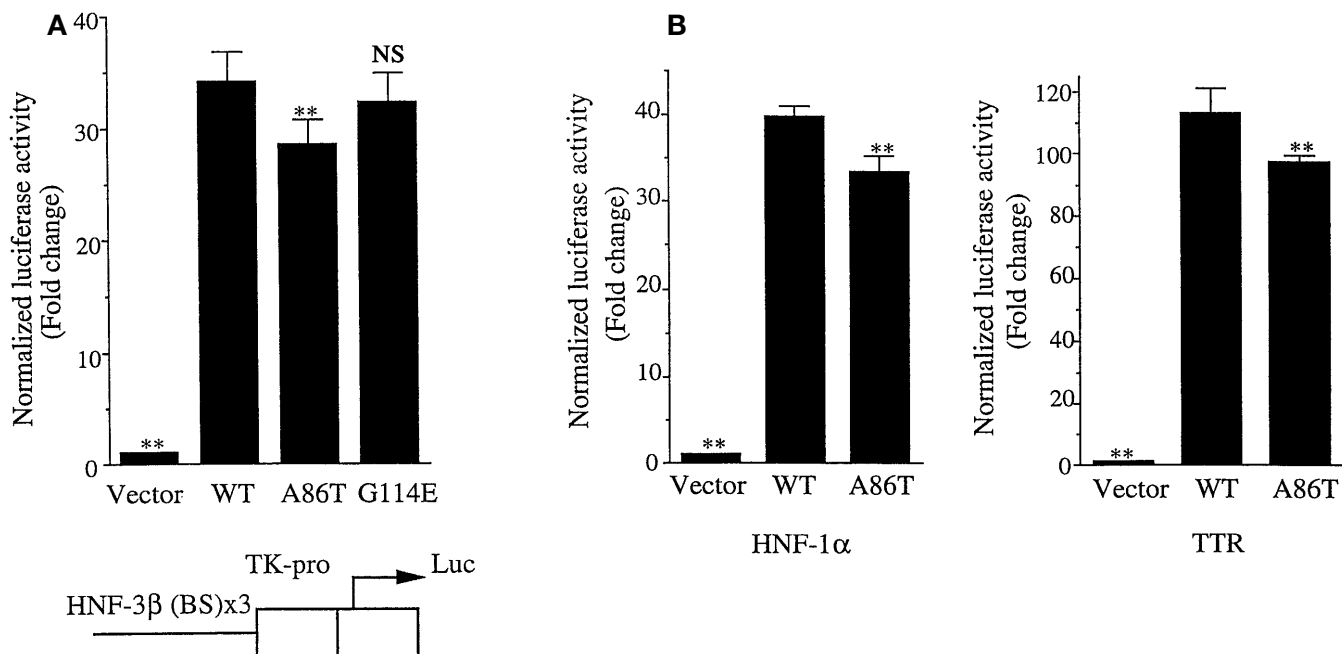
Fig. 1 A, B. Transactivation activities of WT and mutant HNF-3β in HeLa cells. Cells were transfected with 100 ng of WT or mutant HNF-3β constructs and 0.5 µg of the reporter genes together with 10 ng of pRL-SV40. **A** pHNF3β-tk-Luc reporter and **B** HNF-1α-pGL3 and TTR-pGL3. The results were from six independent experiments; means ± SEM, $*p < 0.05$, $**p < 0.01$

diabetes is not known. Recent functional studies have, however, shown that mutant HNF-1α proteins with modestly impaired transactivation activity are associated with a mild form of diabetes (late onset and low penetrance) rather than MODY [8]. The late onset of diabetes observed in subjects with the A86T mutation could be a consequence of the relatively mild nature of the A86T-HNF-3β. Alternatively, this mild mutation could be one of several polygenic factors contributing to the development of Type II diabetes.

This study indicates that mutations in the *HNF-3β* gene are not a common cause of late-onset Type II diabetes in Japanese subjects although the results apply only to the coding region of the *HNF-3β* gene. We also showed that the A86T mutation in the *HNF-3β* gene might be involved in the development of late-onset Type II diabetes in a small group of subjects in this cohort.

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