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Short communication

Three new mutations in the hepatocyte nuclear factor-1α gene in Japanese subjects with diabetes mellitus: clinical features and functional characterization

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Abstract

Aims/hypothesis. Mutations in the *hepatocyte nuclear factor-1α* gene are a common cause of the type 3 form of maturity-onset diabetes of the young. We examined the clinical features and molecular basis of hepatocyte nuclear factor-1α (HNF-1α) diabetes.
Methods. Thirty-seven Japanese subjects with early onset Type II (non-insulin-dependent) diabetes mellitus and 45 with Type I (insulin-dependent) diabetes mellitus were screened for mutations in this gene. Functional properties of mutant HNF-1α were also investigated.
Results. Three new mutations [G415R, R272C and A site of the promoter (+ 102G-to-C)] were found. Insulin secretion was impaired in the three subjects. Insulin and glucagon secretory responses to arginine in the subject with the R272C mutation were also diminished. Molecular biological studies indicated that the G415R mutation generated a protein with about 50 % of the activity of wild-type HNF-1α. The R272C mutation had no transactivating or DNA binding activity and acted in a dominant negative manner. The + 102 G-to-C mutation in the A site of the promoter activity was associated with an increase in promoter activity and it had 42–75 % more activity than the wild-type sequence.
Conclusion/interpretation. Mutations in the *HNF-1α* gene may affect the normal islet function by different molecular mechanisms. [Diabetologia (1999) 42: 621–626]

Keywords MODY, HNF-1α, insulin, arginine, mutation.

Recent studies have shown that heterozygous mutation in the gene encoding hepatocyte nuclear factor-1α (HNF-1α) is a cause of maturity-onset diabetes of the young (MODY3) [1]. Mutations in the *HNF-1α* gene have also been found in patients diagnosed with Type II (non-insulin-dependent) and Type I (insulin-dependent) diabetes mellitus [1, 2, 3]. We have recently identified that the most common *HNF-1α* mutation P291fsinsC is a dominant negative mutation [4]. There is, however, no information about the function of other mutant proteins. To gain a better understanding of the clinical features and molecular basis of HNF-1α diabetes, we screened Japanese subjects with early onset Type II diabetes and Type I diabetes for mutations in this gene and analyzed the functional properties of mutant HNF-1α.

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Abbreviations: MODY, maturity-onset diabetes of the young; *HNF-1α*, hepatocyte nuclear factor-1α; UCPR, urinary C-peptide; OHA, oral hypoglycaemic agent; EMSA, electrophoretic mobility shift assay; TTR, transthyretin; ICA, islet cell antibody; GAD, glutamic acid decarboxylase; WT, wild type.

Subjects and methods

Subjects and screening of HNF-1α gene. We studied 37 unrelated Japanese subjects with early onset Type II diabetes (diagnosis 35 years of age or less, range 14–35 years) including seven

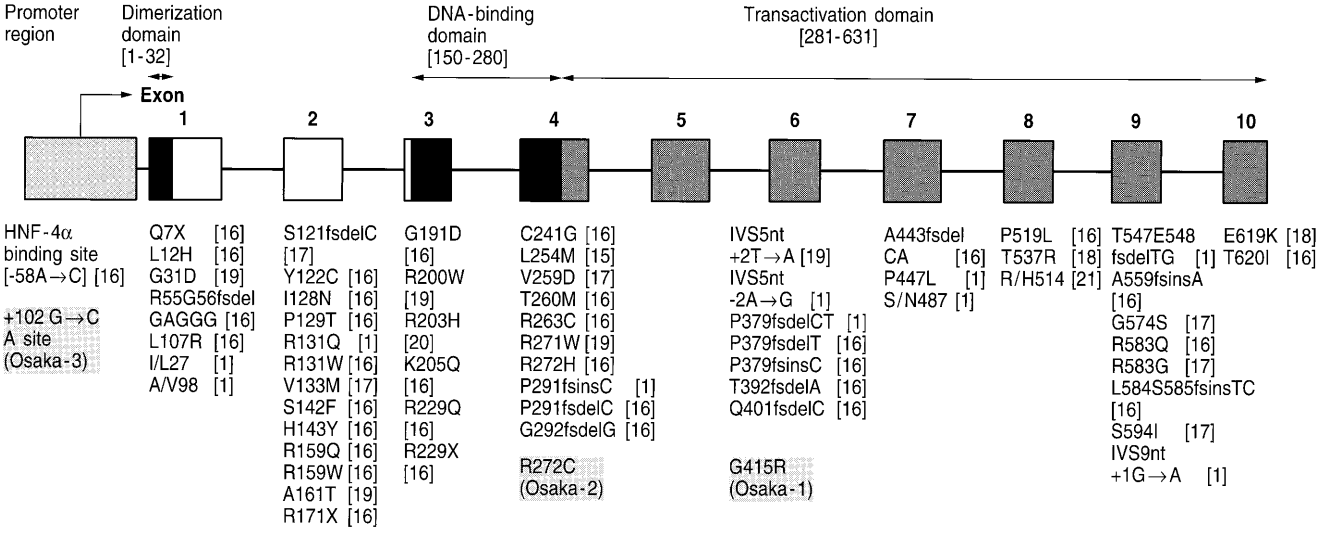

Fig. 1. Summary of mutations in the *HNF-1α* gene. [1, 15–21]

with a clinical diagnosis of MODY and 45 unrelated Japanese subjects with Type I diabetes. Type I diabetes was diagnosed on the basis of proness to ketoacidosis and dependency on insulin treatment due to absolute insulin deficiency, and the subjects fulfilled the criteria of the National Diabetes Data Group for Type I diabetes [5]. Average urinary C-peptide (uCPR) in 35 Type I diabetic patients whose data were available were 2.48 ± 2.35 nmol/24 h (mean ± SD), confirming the existence of insulin dependency in the group. Informed consent was obtained from all participants according to the Helsinki Declaration. Ten exons and the minimal promoter region were screened for mutations [1].

Arginine stimulation test. The arginine stimulation tests were done by the intravenous infusion of 5 g of arginine (Morishita Roussel, Osaka, Japan) in 1 min. Blood samples were obtained at 0, 2, 3, 5, 7, 10 and 20 min. Six healthy subjects (age, 36 ± 4; BMI, 22.2 ± 3.6; HbA_{1c}, 4.8 ± 0.3) (mean ± SD) were used as control subjects.

Construction of plasmids, transfection and Western blot analysis. Human HNF-1α cDNA was subcloned into pcDNA3.1/HisC (Invitrogen, Carlsbad, Calif., USA) and RIP vector [4]. R272C and G415R mutations were introduced by in vitro mutagenesis. A 251 bp of human *transthyretin* (*TTR*) gene promoter fragment containing HNF-1α binding site (–218 to + 33 relative to the cap site) was amplified by PCR and subcloned into pGL3-basic (Promega, Madison, Wis., USA). The sequences of the constructs were confirmed.

MIN6 and HeLa cells were transfected with the indicated amounts of expression vectors together with *TTR* reporter gene and the transactivation activities were measured after 48 h [4]. Western blot analysis was done using anti-Xpress antibody (Invitrogen, DeSchelp, The Netherlands) [4].

Electrophoretic mobility shift assay (EMSA). In vitro translated proteins were incubated with the ^{32}P-labelled oligonucleotide containing HNF-1α binding site of the *TTR* gene (5′-TATGGGTTACTTATTCTCTCTTT-3′) and the DNA-protein complexes were analysed on 5% polyacrylamide gels. The anti-HNF-1α antiserum was prepared after immunizing a rabbit with the synthetic peptide (PTKQVFTSDTEASSESGLHT – amino acid residues 538 to 557) and used for supershift experiments.

Promoter activity assay and EMSA of the A-site mutation + 102G-to-C. The promoter region of the *HNF-1α* gene was amplified from Osaka-3 subject using P primers [6]. A 325 bp of SmaI fragment (–129 to 196 relative to the cap site) which includes wild-type (WT) and mutant A site was subcloned into pGL3-basic. MIN6 and HepG2 cells were transfected with 1 μg of WT or mutant HNF-1α promoter-pGL3. Two μg of nuclear proteins were incubated with the ^{32}P-labelled oligonucleotide containing WT A site sequence (5′-ctaGGCTAGTGGGGTTTTG*G*GGGGGGCAGTGGGTGCAAGG-3′) or mutant A site (5′-ctaGGCTAGTGGGGTTTTG*C*GGGGGCAGTGGGTGCAAGG-3′) and analysed as described above.

Results

Identification of novel mutations. We identified two new missense mutations G415R (transactivation domain) and R272C (DNA binding domain) and a conserved G to C replacement in the A site of the promoter (designated + 102 G to C, relative to the transcription start site) (Fig. 1). These three mutations were not found in 100 non-diabetic Japanese subjects.

Clinical and biochemical profiles of subjects with mutations.

Subject Osaka-1 – G415R mutation. This subject is a 50-year-old woman. She was diagnosed with Type I diabetes at 25 years of age and was treated with insulin (BMI = 22.2). At 33 years, her average uCPR was 2.98 nmol/24 h (normal range, 14.9 to 38.7 nmol/24 h) and the result of the OGTT indicated the impaired insulin secretory response to glucose. Islet cell antibody (ICA) and antibody to glutamic acid decarboxylase (GAD) were negative. Her present HbA_{1c} level is 7.6% and nonproliferative diabetic retinopathy is being observed. Both parents are dead.

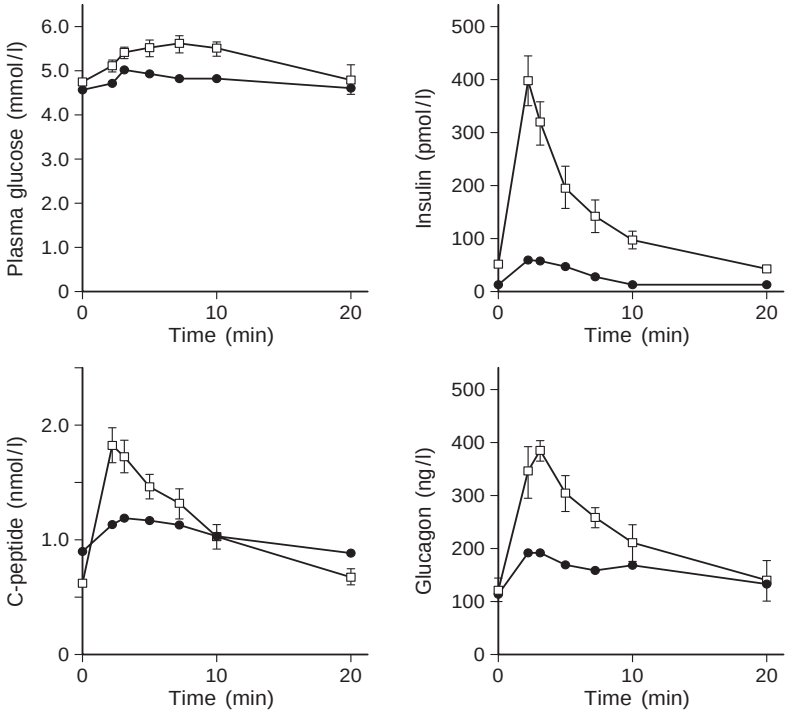

Fig. 2. The effect of arginine on plasma concentrations of glucose, insulin, C-peptide and glucagon. □, control subjects ($n = 6$) (mean ± SEM) ; ●, patient with R272C mutation

Serum IGF-1 concentration, which was decreased in the *HNF-1α* null mice [7], was normal. General hepatic and renal functions were normal.

Subject Osaka-2 – R272C mutation. This subject is a 46-year-old man (BMI = 17.3). He was diagnosed with Type II diabetes at 30 years of age and was treated with an oral hypoglycaemic agent (OHA). As his father and all four of his siblings had diabetes, the form of his diabetes was considered to be MODY. He has a proliferative diabetic retinopathy, diabetic nephropathy and neuropathy. Average uCPR was 4.87 nmol/24 h. C-peptide response to glucagon was also impaired (ΔCPR = 0.46 nmol/l/5 min, normal 1.75 ± 0.32). The effect of the nonglucose secretagogue arginine was investigated (Fig. 2). There was no difference in the initial plasma glucose concentration which affects the arginine-induced insulin secretion [8] between the patient and control subjects. Insulin, C-peptide and glucagon responses were notably impaired, suggesting the presence of alpha-cell as well as beta-cell dysfunction.

The liver isozyme of alkaline phosphatase was increased in this subject (357 U/l, normal range 69 to 185). Computer tomography imaging showed a stone in the gallbladder but no other abnormalities were observed in other organs, including the liver.

Subject Osaka-3 with A site mutation + 102 G to C. The subject is a 43-year-old man. He was diagnosed with diabetes mellitus at 29 years of age and treated with an OHA. After 1 year, proness to ketosis was observed and insulin therapy was introduced with the diagnosis of Type I diabetes. ICA and IAA was negative and uCPR was 0.99 nmol/24 h. His present HbA_{1c} level is 6.3 % and there are no diabetic complications. The result of genotyping of his nondiabetic son was normal. His brother and parents were not diabetic by interview and they did not consent to OGTT or genotyping. A paternal uncle was diagnosed with Type II diabetes at 40 years of age but he was not available for genotyping.

Functional analysis of G415R and R272C. The expression levels of wild type (WT) and mutant proteins in HeLa cells were similar (Fig. 3A). The transactivation activity of G415R was 57 % of that of WT in HeLa cells (Fig. 3B), indicating that this mutation leads to a loss of function. In contrast, R272C had no transactivation activity. MIN6 cells transfected with a R272C-*HNF-1α* construct had decreased luciferase activity compared with empty vector (Fig. 3C). Increasing amounts of the R272C-HNF-1α inhibited the luciferase activity up to 47 % of the control in a dose-dependent manner (Fig. 3D), suggesting that R272C functions in a dominant negative manner.

The binding of WT and mutant HNF-1α to target DNA was assayed by EMSA. The expression levels of in vitro translated proteins were similar (data not shown). R272C-HNF-1α (mutation in the DNA binding domain) could not bind to the target sequence (Fig. 3E). The DNA binding activity of the G415R

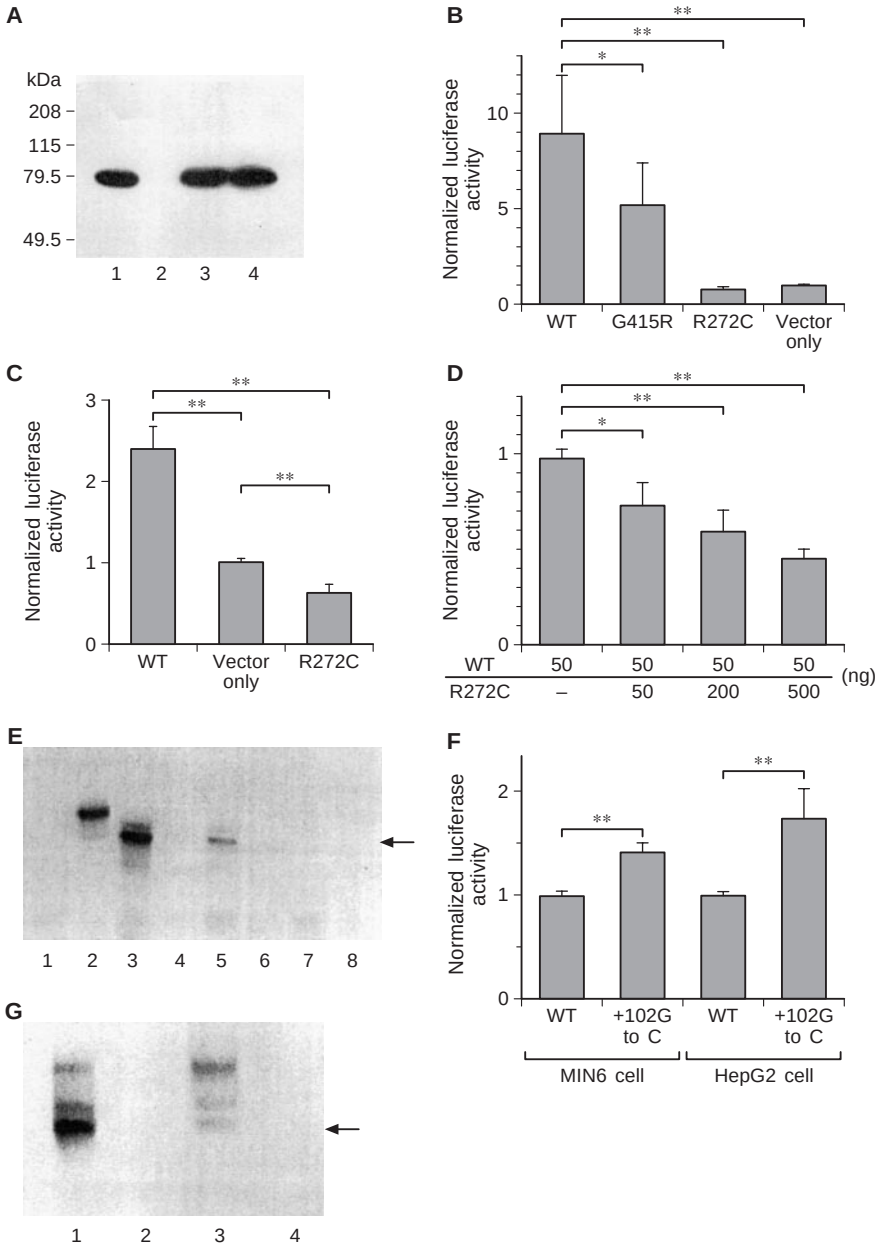

Fig. 3 A–G. Functional properties of G415R and R272C mutant HNF-1α and + 102 G to C mutant promoter. (**A**) Expression of WT and mutant HNF-1α in HeLa cells. 8 μg of pcDNA3.1/HisC expression vectors were transfected and Western blot analysis was done. Lane 1, WT; lane 2, vector only; lane 3, G415R; lane 4, R272C. (*B*) Transcriptional activity of TTR in HeLa cells. 200 ng of WT, G415R, R272C-HNF-1α-pcDNA3.1/HisC and empty vector were transfected with 500 ng of TTR-reporter gene and 10 ng of pRL-SV40 DNA. Luciferase activity was normalized by the activity of pRL-SV40. (**C**) Transcriptional activity of TTR in MIN6 cells. 50 ng of WT, R272C-HNF-1α-RIP and empty vector were transfected with TTR-reporter gene and pRL-SV40. (**D**) 50 ng of WT-HNF-1α-RIP was transfected alone or with increasing amounts (50, 200, 500 ng) of the R272C-HNF-1α-RIP expression vector. The total amount of DNA added was adjusted to 1.15 μg by empty vector. (**E**) Binding abilities of G415R and R272C mutant HNF-1α. EMSA analysis. Lane 1, synthesized protein from empty vector; lane 2, 3 and 4, synthesized protein from WT-HNF-1α cDNA; lane 5 and 6, synthesized protein from G415R-HNF-1α cDNA; lane 7 and 8, synthesized protein from R272C-HNF-1α cDNA. Lane 2, supershift analysis with anti-HNF-1α antiserum. Lane 4, 6 and 8, 50 molar excess of unlabelled probe was used as a competitor. (**F**) Promoter activity assay of WT and + 102 G to C mutant promoter in MIN6 cells and HepG2 cells. (**G**) EMSA analysis. Nuclear proteins of MIN6 cells were incubated with ^{32}P-labelled WT-A site and mutant A site oligonucleotide. Lane 1 and 2, nuclear proteins of MIN6 cells incubated with ^{32}P-labelled WT A site oligonucleotide; lane 3 and 4, nuclear proteins of MIN6 cells incubated with ^{32}P labelled mutant A site oligonucleotide. Lane 2 and 4, excess of unlabelled probe was used as a competitor. The results shown here are the mean of 3–6 experiments. Standard deviations are shown as error bars. * indicates $p < 0.05$. ** indicates $p < 0.01$

(mutation in the transactivation domain) was decreased by 62 %, suggesting that this mutation also affects DNA binding.

Effect of A site mutation + 102G-to-C on promoter activity. The mutant promoter construct showed 1.4-fold and 1.8-fold increased activity in MIN6 and HepG2 cells, respectively (Fig. 3F). Nuclear protein extracts from HepG2 and MIN6 cells bound to WT-A site and the binding was blocked by the addition of excess competitor (Fig. 3G). In contrast, the binding ability was reduced by 89 % when the mutant A site oligonucleotide was incubated with the nuclear proteins (Fig. 3G), suggesting that this one base substitution in the region affects DNA-protein interaction.

Discussion

We found three novel mutations in the *HNF-1α* gene. Mutations were found in 4.4 % of Type I diabetic patients as reported previously [3]. Since these patients lacked immunological markers such as ICA and GAD, they were considered to be cryptic MODY rather than autoimmune Type I diabetes.

We investigated the effects of nonglucose secretagogue arginine on insulin and glucagon secretion in a subject with a R272C mutation. Both insulin and glucagon responses to arginine were reduced (Fig. 2). Such diminished insulin and glucagon responses were also observed in the subjects with Q268X mutation in the *HNF-4α* gene [9]. Hyperfunction rather than hypofunction of the α-cell is usually observed in diabetic patients [10, 11]. These data suggest that both MODY3 and MODY1 are associated with defects in alpha-cell and beta-cell function.

The mutation found in the A site of HNF-1α promoter is the first to be described in this region of the gene. A study showed that an unidentified protein factor binds to this region and that deletion of the A site region is associated with an increase in expression of HNF-1α [12]. Functional studies suggest that the + 102G-to-C mutation possibly leads to increased promoter activity and thus might lead to higher than normal levels of HNF-1α protein and activity. Another study reported that overexpression of HNF-1 negatively regulates the expression of HNF-4-dependent genes that lack HNF-1 binding sites in the promoter region [13]. Mutation of *HNF-4α* gene is a cause of MODY1 [14]. Repression of HNF-4 dependent genes by overexpression of HNF-1α is a possible explanation for the reason of diabetes in the subject. We identified that G415R is a loss-of-function mutation whereas R272C is a dominant negative mutation. These data suggest that both increased as well as decreased levels of HNF-1α activity may have pathophysiological consequences with respect to normal beta-cell function by altering the expression of the target genes in pancreatic beta-cells. The identification of additional families with mutations in the A site and characterization of the effects will clarify this issue. Finally, the R272C mutation was associated with a more progressive and severe form of diabetes than G415R, suggesting that there might be differences in clinical features of patients with different types of mutations.

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