Brief Genetics Report

Identification of Mutations in the Hepatocyte Nuclear Factor-1α Gene in Japanese Subjects With Early-Onset NIDDM and Functional Analysis of the Mutant Proteins

Shirou Yamada, Hideaki Tomura, Hidekazu Nishigori, Kimie Sho, Hiroyo Mabe, Noritaka Iwatani, Toru Takumi, Yoshihiko Kito, Naoki Moriya, Koji Muroya, Tsutomu Ogata, Kazumichi Onigata, Akihiro Morikawa, Ituro Inoue, and Jun Takeda

Maturity-onset diabetes of the young (MODY) is a monogenic form of NIDDM with onset usually before 25 years of age (1). It has been shown that the third type of MODY (MODY3) results from mutations in the gene encoding hepatocyte nuclear factor (HNF)-1α (2), a homeodomain-containing transcription factor (3), which was originally identified in liver and is also expressed in some other tissues, including pancreatic β-cells. Recent screening for mutations in the HNF-1α gene in racially different groups of patients with MODY has shown that defects in this gene are a common cause of MODY in France (25–50%), U.K. (73%), and Japan (~20%) (4–6). Screening of German patients who presented with NIDDM before 35 years of age and had a first-degree relative with NIDDM also identified mutations in 35% of the subjects examined (7), suggesting that early-onset NIDDM in Germany may be mostly a monogenic form of MODY. On the other hand, our previous screening of 60 Japanese subjects with early-onset (<35 years of age) NIDDM, 45 of whom had first-degree relatives with NIDDM, found no mutations in this gene (8), suggesting that most of the early-onset NIDDM in Japanese individuals could be polygenic rather than monogenic. Since the phenotype of MODY3 includes high penetrance of impaired insulin secretion and often affects children at school age, MODY3 may be more frequent in the younger generation in Japan.

In this study, we examined 25 nonobese subjects (BMI <23 kg/m^2) with NIDDM whose glycosuria was first noticed by annual school health examination before 15 years of age, regardless of the family history of diabetes; most of the NIDDM subjects (55 of 60) previously studied were diagnosed after 15 years of age (8). NIDDM was diagnosed by 1.75 g/kg oral glucose tolerance test. Of the patients, 12 are currently being treated with a small amount of insulin (3–12 U/day), and 11 with diet. No therapy has been prescribed for two subjects.

The HNF-1α gene of these subjects was screened for mutations by direct sequencing of the polymerase chain reaction (PCR) products as described (9), resulting in identification of four missense mutations of L12H, K158N, R159Q, and R203C, and one nonsense mutation of R229X (Fig. 1*A*). R203C and K158N are novel mutations. L12H, R159Q, and R229X were previously identified in other MODY families (5–7). Four of the subjects have primary relatives with NIDDM or impaired glucose tolerance (Fig. 1*B*). The parents of the other subject, Y01, were not diabetic by interview (oral glucose tolerance test could not be performed on these subjects). Because Y05 has bilineal inheritance of diabetes, other genetic determinants may also be involved in the development of diabetes in this family.

To analyze the functional properties of the mutant proteins, a luciferase reporter assay was performed using the GLUT2 gene promoter and mutant constructs. Mutations were generated from the wild-type cDNA by PCR-based site-directed mutagenesis and cloned into the expression vector pCMV-6b. The promoter region (nucleotide –1296/+312) of the human GLUT2 gene, which contains an HNF-1α recognition site (10), was cloned into the pGL3-Basic reporter vector (Promega, Madison, WI) to generate the pGL3-GT2. MIN6 cells (11) were transfected with the liposomal DOTAP/nucleic acid mixture including 5 μg of DOTAP, i.e., N-[1-(2,3-dioleoyloxy) propyl]-N,N,N,-trimethylammonium methylsulfate (Boehringer Mannheim, Mannheim, Germany), 333 ng of pGL3-GT2, 12–96 ng of test plasmid, and 17 ng of pRL-SV40 (*Renilla* luciferase) as an internal control. Luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay System (Promega).

From the Laboratory of Molecular Genetics (S.Y., H.T., H.N., K.S., I.I., J.T.), Department of Cell Biology, Institute for Molecular and Cellular Regulation; and the Department of Pediatrics (S.Y., K.O., A.M.), Gunma University, Maebashi, Gunma; the Department of Child Development (H.M., N.I.), Kumamoto University Medical School, Kumamoto; the Department of Pediatrics (T.T., Y.K.), Nishiwaki Municipal Hospital, Hyogo; the Department of Pediatrics (N.M.), Tendo City Hospital, Yamagata; and the Department of Pediatrics (K.M., T.O.), Keio University School of Medicine, Tokyo, Japan.

Address correspondence and reprint requests to Jun Takeda, MD, DMSci, Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15, Showa-machi, Maebashi, Gunma 371-8512, Japan. E-mail: jtakeda@akagi.sb.gunma-u.ac.jp.

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S.Y. and H.T. contributed equally to this work.

HNF, hepatocyte nuclear factor; MODY, maturity-onset diabetes of the young; PCR, polymerase chain reaction.

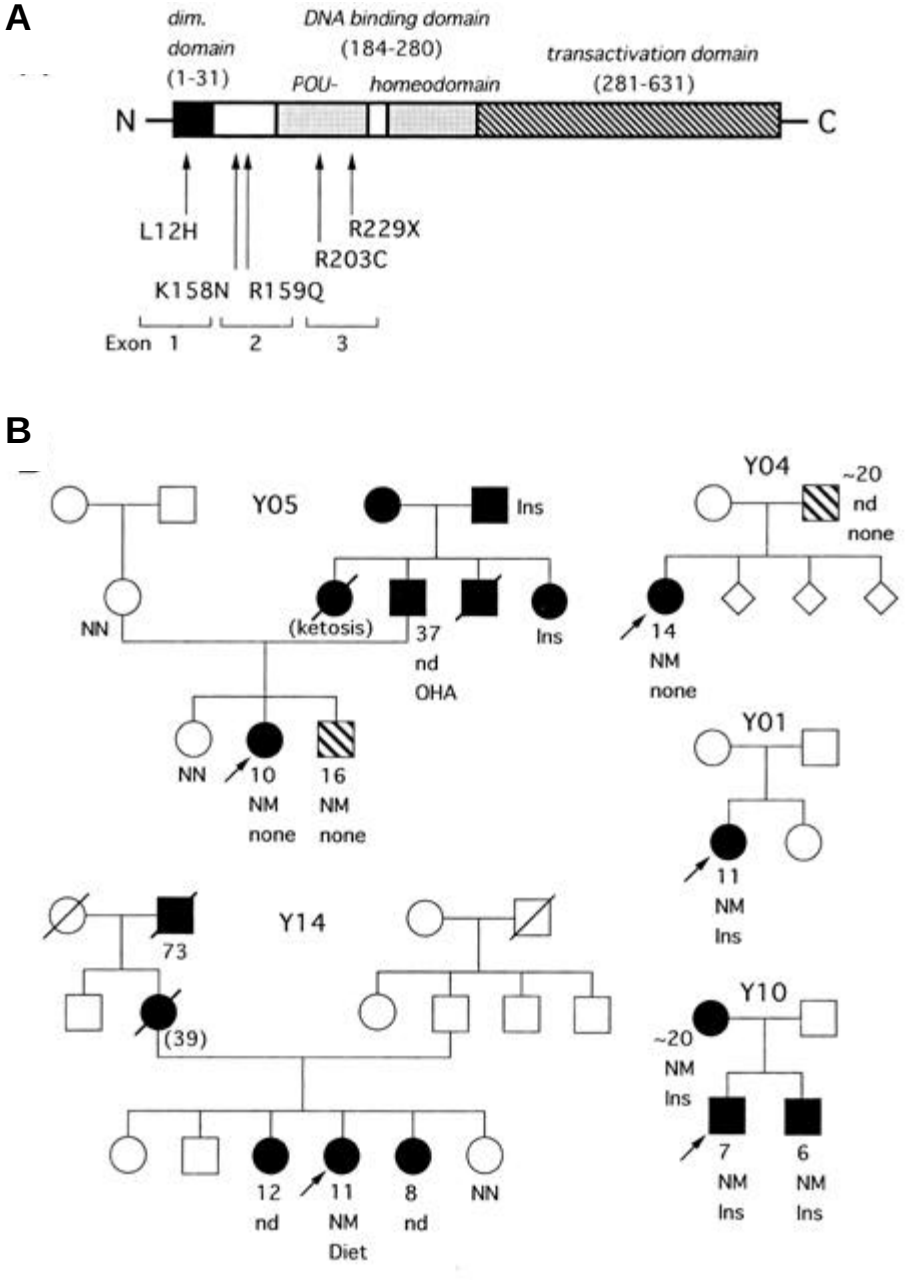

FIG. 1. ***A*****: Schematic representation of the structure of HNF-1α showing different functional domains of the protein and the relative positions of five mutations. The human gene (*TCF1*) encoding HNF-1α consists of 10 exons. The nucleotide alterations were observed in exons 1, 2, and 3. The nonsense mutation of R229X would generate a mutant truncated protein of 228 amino acids.** ***B*****: Pedigrees of families with mutations in the HNF-1α gene. Subjects with NIDDM and impaired glucose tolerance are noted by black and hatched symbols, respectively, and nondiabetic subjects by open symbols. The age at diagnosis of diabetes, if known, and the genotype are indicated below the symbol. Probands are indicated by the arrows. Proliferative diabetic retinopathy was observed only in subject Y14. The mother of Y14 died at the age of 39 years from renal failure. Y10, L12H (CTC-CAC); Y04, K158N (AAG-AAT); Y05, R159Q (CGG-CAG); Y01, R203C (CGT-TGT); Y14, R229X (CGA-TGA). Ins, insulin; M, mutant allele; N, normal allele; OHA, oral hypoglycemic agent.**

The wild-type protein stimulated transcription from the GLUT2 gene promoter and generated a significant increase in luciferase reporter activity (Fig. 2*A*). R229X, which lacks an entire transactivation domain in the COOH-terminus, had almost no activity over a wide range of DNA concentrations. The mutations at the neighboring sites of K158N and R159Q showed a similar pattern of significantly reduced activity compared with that of the wild type. In contrast, although the decreased activity was also observed in L12H and R203C at low DNA concentrations (<48 ng), activity higher than that of the wild type was observed when the amounts of transfected DNA were increased.

Because the mutant proteins, except L12H, retain the intact dimerization domain, there is a possibility that they interact with wild type to modify the normal activity. When equimolar amounts of expression plasmid DNA for the wild type and each mutant were cotransfected, no reduction of the normal activity was observed (Fig. 2*B*). However, when a higher DNA amount (96 ng, molar ratio 1:4) was used, a change in the levels of transcription was observed in K158N and R203C. K158N significantly decreased the wild-type activity, suggesting that this mutation might be a weak dominant-negative regulator. The hypertransactivation activity of R203C that was observed at the DNA amount of 96 ng was diminished to the levels of wild type in the presence of wild type. Accordingly, the various patterns of functional abnormalities associated with the HNF-1α mutations may underlie the differing severities of the insulin secretion defects that are seen in patients with MODY3 (4).

To determine if the distribution of the mutant proteins within the cells could be involved in the modified transcriptional activity of the mutant proteins, we examined localization of the epitope-tagged proteins expressed in MIN6 cells by fluorescent microscopy. The cells were transiently trans-

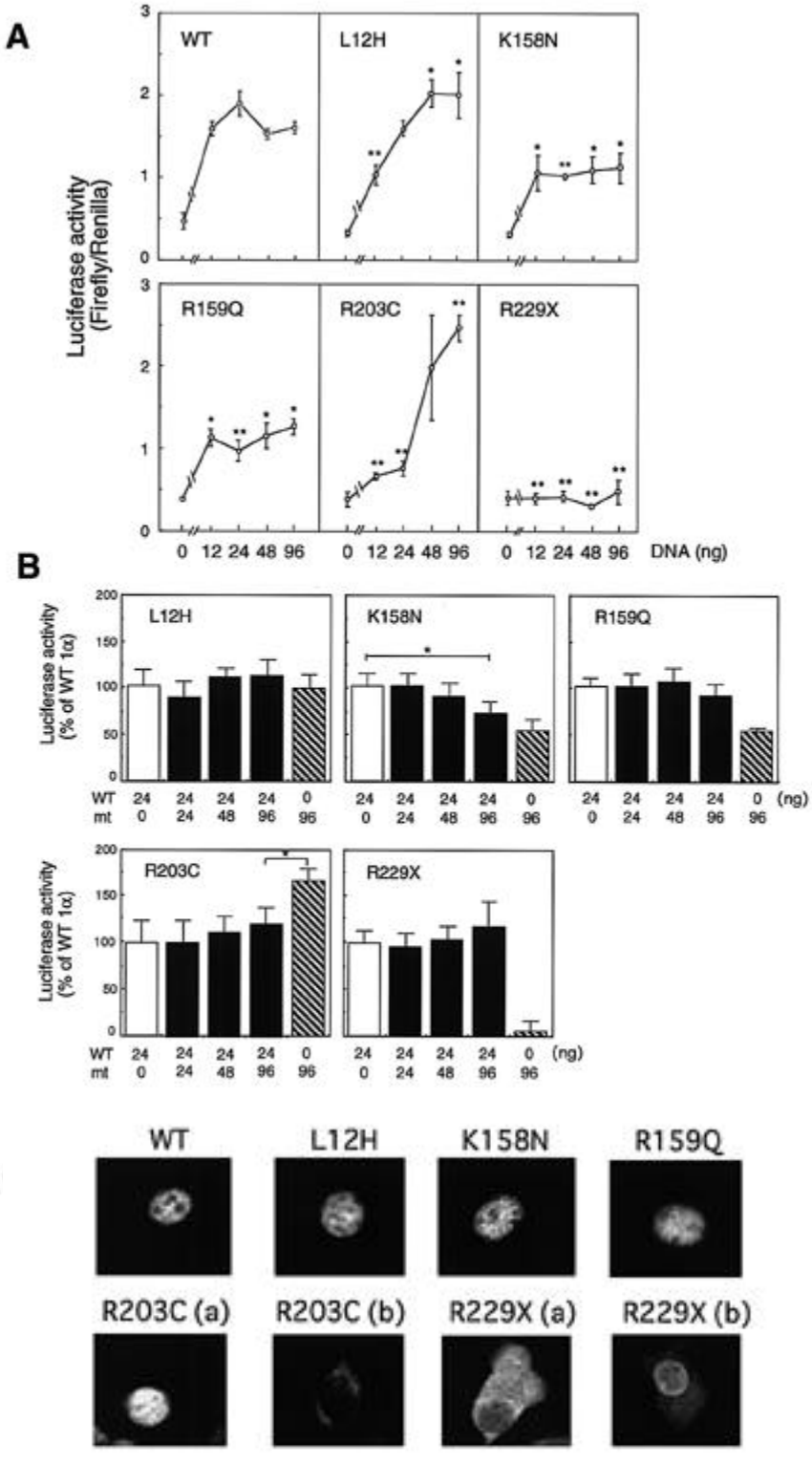

FIG. 2. ***A*: Transactivation activity of human HNF-1α and mutant proteins. pGL3-GT2 and each test plasmid were co-transfected into MIN6 cells. MIN6 cells were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 15% fetal calf serum. The relative activity (firefly luciferase/*Renilla* luciferase) of each construct at 0, 12, 24, 48, and 96 ng was measured by 3–4 independent experiments. Each experiment showed similar results, and a representative result is shown. Means ± SD are shown. *$P < 0.05$, **$P < 0.01$. WT, wild type. *B*: Transient coexpression of wild-type and mutant proteins. pGL3-GT2, wild type and each test plasmid were cotransfected into MIN6 cells. The effect of increasing amounts (0, 24, 48, 96 ng) of each test plasmid on wild type was examined. Open and hatched bars indicate the activity of the wild type and each mutant alone, respectively. *$P < 0.05$. WT, wild type; mt, mutant. *C*: Intracellular localization of wild-type and mutant proteins in MIN6 cells. The cells were transiently transfected with the expression plasmid encoding wild-type and mutant proteins with the hemagglutin protein (HA) epitope tag (YPYDVPDYA). Immunohistochemical analysis was performed using anti-HA antibody. Fluorescein isothiocyanate–conjugated anti-rabbit IgG was used as secondary antibody. A typical pattern of staining from each experiment is shown. Localization of mutant and wild-type proteins in the cells was analyzed by immunofluorescence microscopy (BX-50; Olympus, Tokyo, Japan). Two different patterns of staining in R203C and R229X are shown as *a* and *b*. Nuclear staining of the wild-type and mutant proteins was confirmed by staining with 4,6-diamidino-2-phenylindole-dihydrochloride.**

fected with the wild-type and mutant proteins. Strong signals were found only in the nucleus in L12H, K158N, and R159Q, as in the wild type (Fig. 2*C*). Interestingly, although clear staining of R203C was found only in the nucleus in most of the cells, a small number showed weak but positive signals around the nuclear membrane on the cytoplasmic face. Signals of R229X, which showed no transactivation activity in the luciferase reporter assay, were observed primarily in the cytoplasm in most of the cells, although some of the slightly stained cells showed signals around the perinuclear membrane on the inside face of the nucleus. The modified localization of R229X might be due to lack of a nuclear localization signal in the COOH-terminal region, which has previously been suggested by COOH-terminal deletion studies (12). Since the residue Arg-203 locates in the middle of the short stretch of basic residues that also possibly specify nuclear tar-

geting (3), the localization of R203C could be affected by the key amino acid change. Such modified localization of R203C and R229X could be involved, at least in part, in the biphasic and null effects, respectively, on transcriptional regulation in the luciferase reporter assay.

In this study, five mutations were identified in the HNF-1α gene in Japanese subjects with early-onset (<15 years of age) NIDDM. Since the frequency of the mutations in this study (20%) is similar to that found in the previous study of Japanese MODY (6), the disease could well be common in this group of subjects with NIDDM diagnosed before 15 years of age, even though most of the NIDDM in Japan diagnosed after 15 years of age could be a polygenic form of diabetes. These results suggest that subjects with early-onset NIDDM, especially those first noticed by glycosuria at school age, should be screened for mutations in the HNF-1α gene in Japanese, regardless of the family history of diabetes.

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