Hepatocyte nuclear factor 1-alpha mutation in normal glucose-tolerant subjects and early-onset type 2 diabetic patients

Dong Mee Lim, M.D.¹, Nam Huh, Ph.D.² and Keun Yong Park, M.D.¹

¹Department of Internal Medicine, Konyang University College of Medicine, Daejon, Korea; ²Samsung Advanced Institute of Technology, Yongin, Korea

Background/Aims: The prevalence of diabetes in Korea is reported to be approximately 10%, but cases of maturity-onset diabetes of the young (MODY) are rare in Korea. A diagnostic technique for autosomal dominant MODY is being actively sought. In this regard, we used a DNA chip to investigate the frequency of mutations of the MODY3 gene (hepatocyte nuclear factor-1α) in Korean patients with early-onset type 2 diabetes.

Methods: The genomic DNA of 30 normal individuals [age, 24.9±8.6 years] and 25 patients with early-onset type 2 diabetes (age, 27±5.9 years) was extracted, and the MODY3 gene was amplified. The amplified DNA was hybridized onto a MODY3 chip, which has oligonucleotides of 15-25 bases, representing wild-type and mutant MODY3 sequences in both forward and reverse orientations, immobilized on its surface.

Results: Among the normal subjects, there was no mutation of MODY3. Among those with early-onset type 2 diabetes, there was one case of MODY3 mutation.

Conclusions: Our results indicate that MODY3 mutations are not rare in Korean early-onset type 2 diabetes patients in Korea and suggest that MODY3 mutations in patients with early-onset type 2 diabetes need to be further evaluated.

Key Words: DNA chips; MODY; Type 2 diabetes mellitus

INTRODUCTION

The prevalence of diabetes in Korea is reported to be approximately 10% and most patients have type 2 diabetes mellitus. Maturity-onset diabetes of the young (MODY) is a heterogeneous form of diabetes characterized by onset at an early age, impaired insulin secretion, and inheritable as an autosomal dominant trait for more than three generations.

MODY has numerous subtypes, depending upon the gene that is involved: MODY1 is associated with chromosome 20; MODY2, with chromosome 7; MODY3, with chromosome 12; MODY4, with chromosome 13; MODY5, with chromosome 17; and MODY6, with chromosome 2. The MODY3 gene encodes hepatocyte nuclear factor (HNF)-1α, which is a homeodomain-containing transcription factor required for tissue-specific expression of a variety of genes in the liver, kidney, pancreas (including the Islets of Langerhans), intestine, stomach, spleen, and thymus. Although mutations of the MODY2 gene (glucokinase) are the most common mutations in French MODY patients, MODY3 (HNF-1α) mutations are the most common mutations in Western and Asian countries. Nevertheless, only a few cases of MODY have been reported in Korea.

Diagnostic techniques for autosomal dominant MODY are currently being sought. In this study, we used a DNA chip to investigate the frequency of MODY3 mutations in Korean patients with early-onset type 2 diabetes.
Table 1. Clinical characteristics and laboratory findings

| Variables               | Normal group (n=30) | Diabetes group (n=25) |
|-------------------------|---------------------|-----------------------|
| Age (years)             | 24.9±8.6            | 27.0±5.9              |
| Gender, male            | 17 (57%)            | 13 (52%)              |
| BMI (kg/m²)             | 22.5±3.0            | 24.0±3.2              |
| SBP (mmHg)              | 121.2±11.1          | 135.6±15.7            |
| DBP (mmHg)              | 70.3±11.3           | 78.0±11.9             |
| Fasting glucose (mg/dL) | 93.8±11.2           | 145.0±70.9            |
| 2-h glucose (mg/dL)     | 128±9.4             | 299.2±90.1            |
| Total cholesterol (mg/dL)| 173.4±48.3         | 170.1±51.0            |
| Triglyceride (mg/dL)    | 124.8±110.7         | 163.6±99.7            |
| HDL cholesterol (mg/dL) | 52.6±11.3           | 40.9±9.7              |
| LDL cholesterol (mg/dL) | 96.6±40.7           | 96.9±48.3             |
| Basal C-peptide (ng/mL) | 2.3±2.5             | 3.6±4.5               |
| *2-h C-peptide (ng/mL)  | 3.8±2.6             | 4.2±2.9               |

SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *level after a 75-g oral glucose tolerance test.

Table 2. PCR primer sequences for the HNF-1α gene

| Site       | Forward       | Reverse         |
|------------|---------------|-----------------|
| Promoter   | TGGCCGTGAGCATCCTCTGCC | GCGTGGGTTGGTTGGCTGTC |
| Exon 1     | CGTGCCCTTGCGAGCAGCGCA | GGGCTCGTTAGGACTGGAGG |
| Exon 2     | CCCTTTGACGAGATCCCAAGCTC | GGATGTTAGGCTCCAGgcc |
| Exon 3     | GCAAGTCTAGGAGAAATGGGA | CCGCATTCTACATTGCACTCC |
| Exon 4     | GGCTCATGGGGTGGACTATTC | CGTGGCCTGTGGGCACATACC |
| Exon 5     | TGCTGTAGGAGCAGACTTGCGC | TACAAGAACGAGCACTACACAGC |
| Exon 6     | CCCCACACAGCTGGCTCCCA | ATCCCCACCACGCTACGAGAC |
| Exon 7     | CAGGCTCTGGCTCAGCTAGC | GGGGCTCGTGCAGCTGAGCAGC |
| Exon 8     | GGCACGTCAGCAGCAGGGG | GGGCAGGAGCAGTACAGGGAG |
| Exon 9     | GGCACGTCACGGCAGCAGGG | GGGCAGGAGCAGTACAGGGAG |
| Exon 10    | GCCTTGTGGCTGCAGTG | GGCATCTGGGTTGGAGTAGAAG |

MATERIALS AND METHODS

Study subjects
Thirty normal individuals [age (mean±SD), 24.9±8.6 years] who had no family history of diabetes and who showed a normal serum glucose level in a 75-g oral glucose tolerance test (OGTT) were enrolled in this study as controls. We also enrolled 25 patients (age, 27±5.9 years) who had been diagnosed with early-onset diabetes before the age of 35 years. The diagnostic criteria for type 2 diabetes followed those of the 57th ADA Conference held in Boston in June 1997. Glucose values of <110 mg/dL (6.1 mmol/L) and <140 mg/dL (7.75 mmol/L) were used as the normal fasting plasma glucose level and normal 2-h glucose level after a 75-g OGGT, respectively. The normal C-peptide level was >0.6 ng/mL. Insulin and glutamic acid decarboxylase auto-antibodies were noted at diagnosis. We measured the height, weight, body mass index (BMI), and blood pressure for each subject, and determined the fasting glucose level, glucose level 2-h after a 75-g OGGT, and insulin and C-peptide levels in both the normal and early-onset type 2 diabetes groups.

Nucleotide sequencing
From each subject, a blood sample (4 mL) was collected into a tube containing EDTA. Genomic DNA was extracted from the whole blood using a genomic DNA extraction kit (Promega, Madison, WI, USA). Single or multiplex PCR amplification of the HNF-1α gene was carried out using 10 different primer sets (Table 2). Direct sequencing of the PCR products was performed using an ABI Dye Terminator Cycling Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions, followed by analysis on an ABI3700 DNA sequencer (Applied Biosystems).

Site-directed mutagenesis
Site-directed mutations were introduced into MODY3 DNA by using a PCR method and primers modified for the specific mutations (Bioneer, Seoul, Korea). The PCR products were inserted into plasmids, which were used to transform bacteria. The plasmid DNA was purified using a commercial kit (Qiagen, Madison, WI, USA).
Dong Mee Lim, et al. HNF-1α mutation in normal and early-onset type 2 diabetes patients

Figure 1. Schematic of microspotting the MODY3 chip.

Figure 2. (A) Results of a MODY3 microarray analysis of DNA from a patient with type 2 diabetes and no HNF-1α promoter mutation. The white rectangle outlines the area of decreased signal intensity owing to the wild-type HNF-1α gene. (B) Results of a MODY3 microarray analysis of DNA from a patient with type 2 diabetes and a HNF-1α promoter mutation. The white rectangle outlines the area of increased signal intensity owing to the mutant HNF-1α promoter sequence.

Valencia, CA, USA), and the mutations were confirmed by direct sequencing.

PCR and hybridization

For PCR, the reaction mixture contained 0.2 μg of either wild-type or mutant DNA, 100 μM of each dNTP (Amersham Biosciences, Uppsala, Sweden), and 100 nM each of five multiplex primer sets: one set for mutations in the promoter region and exons 2, 4, and 7, and four sets for mutations in exons 1, 3, 5, 6, 8, 9, and 10. The PCR conditions were 35 cycles of 95°C for 30 s, 64°C for 15 s, and 72°C for 3 min. The PCR product was purified using a Qiagen kit, and the purified PCR product (A260/A550 = 1.0-3.5) was used for hybridization.

The PCR product was diluted to 150-187.5 nM, mixed with Cy3-labeled tag oligonucleotide, denatured at 94°C for 5 min, cooled on ice, and then mixed with 30 μL of hybridization buffer [6×SSPE (0.15 M NaCl, 10 mM Na2HPO4, 1 mM EDTA, pH 8.0), 0.1% Triton X-100, 25% formamide], A HybriWell (Molecular Probes, Eugene, OR, USA) was sealed to a Gen-Spector® MODY3 chip (2.54 x 7.62 cm; Samsung, Yongin, Korea), which has oligonucleotides of 15-25 bases, representing wild-type and mutant probes in both forward and reverse orientations, immobilized on its surface. Sixty microliters of PCR fragment-hybridization buffer mixture were added to the HybriWell through a hole in the sealer, the air bubbles were removed, and the hole was covered with a stopper. Hybridization was performed at 48°C for 4 h, followed by washing with 3×SSPE/0.005% Triton X-100 buffer and then 1×SSPE/0.005% Triton X-100 buffer. The chip was dried for 15 min at room temperature.

The hybridization signals were detected by scanning the array using a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA), and digitized images were produced using GenePix Pro software (Axon) (Figure 2). The data were analyzed using a module of the GenSpector® TMC-1000 system (Samsung). The probability that a sample represented a specific mutant was calculated using an algorithm based on an MA-plot determined in preliminary
experiments using wild-type and mutagenized DNA samples, A sample was considered to harbor a particular mutation if the probability that the sample belonged to the specific mutant was $>99.9\%$. A sample was considered wild-type for the mutation if the probability that the sample belonged to the specific mutant was $<0.001\%$. The call rate was 95\%, and the remaining 5% of the spots were in the gray zone, The accuracy was 99.7\%, and the reproducibility was 99.8\%.

RESULTS

In the normal group ($n=30$), both the fasting serum glucose level ($93.8\pm11.2$ mg/dL) and the 2-h glucose level after a 75-g OGTT ($95.6\pm20.3$ mg/dL) were normal. The insulin level was $7.7\pm2.3\mu$U/mL when fasting and $33.4\pm25.3\mu$U/mL at 2 h after a 75-g OGTT; the respective C-peptide levels were $2.3\pm2.53$ and $8.5\pm4.6$ ng/mL. These values are consistent with normal insulin secretion.

In the early-onset type 2 diabetes patients ($n=25$) who were treated with oral anti-diabetic agents, the basal C-peptide level was $3.6\pm4.55$ ng/mL, and the C-peptide level at 2 h after a 75-g OGTT was $7.8\pm8.1$ ng/mL (Table 1).

We used DNA chips to detect mutations of the HNF-1α gene in patients and normal controls. No mutations were detected in any control subject. One patient in the diabetic group had a mutation (Figure 2). Direct sequencing of the HNF-1α mutation in this patient revealed a C substituted for a G in the promoter region of the gene (5'-ctagGCTAGTGGGTTTTCGCG GGCG GGTGGTGCAAGG-3'). The patient with the mutation, a woman, had been diagnosed with diabetes at 25 years of age. Her plasma glucose levels were 151 and 273 mg/dL prior to and 2 h after the intake of 75 g of glucose, respectively. Her serum basal C-peptide level was $3.83$ ng/mL, and her BMI was $22$ kg/m² at the time of diagnosis. She is now controlling her diabetes with diet, physical activity, and metformin. Her hemoglobin A1c level is being maintained at 6.5%. Her grandmother and father had diabetes mellitus, but their blood samples were not available for genotyping.

DISCUSSION

PCR techniques for genetic analysis were developed in the early 1990s and have enabled the identification of genes involved MODY. DNA microarray technology is a new automated method for obtaining large amounts of information in a few hours. The quick and accurate diagnosis of disorders caused by genetic mutations is an expected clinical application of DNA microarray technology. The development and application of DNA chips for specific genetic disorders would form the foundation of personal preventive medicine. A DNA chip could be used to rapidly, conveniently, and accurately predict the genetic predisposition of an individual. With classical methods of gene sequencing analysis, identifying MODY mutations takes more than 3 days; however, with the MODY3 chip (Figure 1), MODY can be diagnosed in only 0.5 days9,11. Using a MODY3 chip, more than 70 reported mutation sites in the HNF-1α gene can be screened with $>99.7\%$ accuracy, using our in-house-developed diagnostic software, GenSpector® Analyser.

MODY accounts for up to 3% of diabetes cases in most Caucasian populations6,14-19. The MODY gene on chromosome 12q has been shown to encode HNF-1α, which had not previously been known to have a role in the functioning of pancreatic β cells. Mutations in HNF-1α (MODY3) are the most common cause of MODY and are thought to be the result of a hotspot for spontaneous mutations in the gene, rather than the result of a single distantly related family mutation. Among families with a HNF-1α mutation, 15% have an insertion of an extra nucleotide (C) in the G tract in exon 4, which leads to a frameshift14,10,20.

The clinical symptoms associated with HNF-1α mutations are markedly different from those associated with glucokinase mutations21. With HNF-1α mutations, the onset of hyperglycemia occurs in adolescence or early adulthood and may be progressive and more severe, with more frequent microvascular complications. In contrast, with glucokinase mutations, hyperglycemia begins in early childhood (from birth) and is mild, with minor age-related deterioration; microvascular complications occur more rarely than with HNF-1α mutations.

Although the prevalence of MODY3 has been reported for many countries, the prevalence of MODY3 in type 2 diabetes has not been reported for Korea. If the prevalence and inheritance of MODY3 in Koreans were known, accurate diagnosis and treatment may be possible, and the risk for MODY3 could be predicted in normal individuals born into families with a history of diabetes. Knowledge of the frequency of MODY3 among Koreans would help in the accurate and early diagnosis of diabetes, thus minimizing diabetic complications and early-onset diabetes.

In the present study with 25 early-onset type 2 diabetes patients, the prevalence of HNF-1α mutations was 4%. There have been three other reports regarding mutations of the HNF-1α gene in Koreans, Lee et al.7 identified a mutation in exon 4 (C900A), which occurred in only one of 69 Korean subjects with early-onset type 2 diabetes; this mutation was silent, although it did cause an amino acid substitution at Pro300, Kim et al.22 identified the HNF-1α R267L mutation in one of 16 unrelated patients who had been diagnosed with type 2 diabetes at $\approx35$ years of age and who had autosomal dominant inheritance of
type 2 diabetes in at least two generations, Shin et al.\(^{23}\) reported that the overall frequencies of HNF-1\(\alpha\) and glucokinase gene mutations were 5 and 2.5%, respectively, in 40 Korean adolescent patients with type 2 diabetes and a strong family history of diabetes.

In our opinion, the prevalence of MODY3 in early-onset type 2 diabetes in Korean patients is 1.4~6.4%. Thus, MODY 3 is not rare in early-onset type 2 diabetes, and MODY mutations in patients with early-onset type 2 diabetes need to be further evaluated.

REFERENCES

1) Kim SM, Lee JS, Lee J, Na JK, Han JH, Yoon DK, Bak SH, Choi DS, Choi KM. Prevalence of diabetes and impaired fasting glucose in Korea: Korean National Health and Nutrition Survey. Diabetes Care 29:226-231, 2006
2) Mendel DB, Crabtree GR. Hepatocyte nuclear factor 1\(\alpha\), a member of a novel class of dimerizing homeodomain proteins, J Biol Chem 266:677-680, 1991
3) Emens LA, Landers DW, Moss LG. Hepatocyte nuclear factor 1\(\alpha\) is expressed in a hamster insulinoma line and transactivates the rat insulin 1 gene. Proc Natl Acad Sci U S A 89:7300-7304, 1992
4) Ellard S. Hepatocyte nuclear factor 1 alpha (HNF-1\(\alpha\)) mutations in maturity-onset diabetes of the young, Hum Mutat 16:377-385, 2000
5) Herman WH, Fajans SS, Ortiz FJ, Smith MJ, Sturis J, Bell GI, Polonsky KS, Halter JB. Abnormal insulin secretion, not insulin resistance, is the genetic or primary defect of MODY in the RW pedigree, Diabetes 43:40-46, 1994
6) Iwasaki N, Oda N, Ogata M, Hara M, Hinokio Y, Oda Y, Yamagata K, Kanematsu S, Oghawara H, Omori Y, Bell GI. Mutations in the HNF-1\(\alpha\)/MODY3 gene in Japanese subjects with early- and late-onset NIDDM, Diabetes 46:1504-1508, 1997
7) Lee HJ, Ahn CW, Kim SJ, Song YD, Lim SK, Kim KR, Lee HC, Huh KB. Mutation in hepatocyte nuclear factor-1\(\alpha\) is not a common cause of MODY and early-onset type 2 diabetes in Korea, Acta Diabetol 38:123-127, 2001
8) ADA International Expert Committee, Report of the Expert Committee on the diagnosis and classification of Diabetes mellitus, Diabetes Care 20:1183-1197, 1997
9) Altman TJ. DNA microarrays in medical practice, BMJ 323:611-615, 2001
10) Maughan NJ, Lewis FA, Smith V. An introduction to arrays, J Pathol 193:3-6, 2001
11) Park GH, Ham OH, Kim KH, Huh N. Oligonucleotide chip for the diagnosis of HNF-1\(\alpha\) mutations, Biosens Bioelectron 21:637-644, 2005
12) Nagan N, O’Kane DJ. Validation of a single nucleotide polymorphism genotyping assay for the human serum paroxonase gene using electronically active customized microarrays, Clin Biochem 34:589-592, 2001
13) Vaxillaire M, Bocci V, Philipp F, Vitouroux C, Terwilliger J, Passa P, Beckman JS, Velho G, Lathrop GM, Froguel P. A gene for maturity-onset diabetes of the young (MODY) maps to chromosome 12q. Nat Genet 9:418-423, 1995
14) Frayling TM, Bulamn MP, Ellard S, Appleton M, Dronsfield MJ, Mackie AD, Baird JD, Kaisaki PJ, Yamagata K, Bell GI, Bain SC, Hattersley AT. Mutations in the hepatocyte nuclear factor 1 alpha gene are a common cause of maturity-onset diabetes of the young in the United Kingdom, Diabetes 46:720-725, 1997
15) Kaisaki PJ, Menzel S, Lindner T, Oda N, Rjasanowski I, Sahm J, Meincke G, Schulze J, Schmelch H, Petzold C, Ledermann HM, Sachse G, Boriraj W, Menzel R, Kerner W, Turner RC, Yamagata K, Bell GI. Mutations in the hepatocyte nuclear factor 1 gene in MODY and early-onset NIDDM: evidence for a mutational hotspot in exon 4, Diabetes 46:539-535, 1997
16) Vaxillaire M, Rouard M, Yamagata K, Oda N, Kaisaki PJ, Boriraj VV, Chevre JC, Bocci V, Cox RD, Lathrop GM, Dussoix P, Philippe J, Timsit J, Charpentier G, Velho G, Bell GI, Froguel P. Identification of nine novel mutations in the hepatocyte nuclear 1 alpha gene associated with maturity-onset diabetes of the young (MODY3). Hum Mol Genet 6:583-586, 1997
17) Hansen T, Elbergher M, Rouard M, Vaxillaire M, Moller AM, Rasmussen SK, Fridberg M, Uhrhammer SA, Holst JJ, Almind K, Echwald SM, Hansen L, Bell GI, Pedersen O. Novel MODY3 mutations in the hepatocyte nuclear factor-1a gene: evidence for a hyperexcessitivity of pancreatic B-cells to intravenous secretagogues in a glucose tolerant carrier of a P447L mutation, Diabetes 46:726-730, 1997
18) Gragnoli C, Lindner T, Cockburn BN, Kaisaki PJ, Gragnoli F, Marozzi G, Bell GI. Maturity-onset diabetes of the young due to a mutation in the hepatocyte nuclear factor-4 alpha binding site in the promoter of the hepatocyte nuclear factor-1 alpha gene, Diabetes 46:1648-1651, 1997
19) Gucksmann MA, Lehto M, Tayber O, Scottti S, Berkeimbier L, Pulido JC, Wu Y, Nig WJ, Fang L, Markel P, Munnelly KD, Goranson J, Orho M, Young BM, Whitacre JL, McMenimen C, Wantman M, Tuomi T, Warram J, Forsblom CM, Carlsson M, Rosenzweig J, Kennedy G, Duyk GM, Thomas JD. Novel mutations and a mutational hotspot in the MODY3 gene, Diabetes 46:1081-1086, 1997
20) Frayling TM, Bulman MP, Appleton M, Hattersley AT, Ellard S. A rapid screening method for hepatocyte nuclear factor 1 alpha frameshift mutations: prevalence in maturity-onset diabetes of the young and late-onset non-insulin dependent diabetes, Hum Genet 101:351-354, 1997
21) Appleton M, Ellard S, Bulman M. Clinical characteristics of the HNF1\(\alpha\) (MODY3) and glucokinase mutations, Diabetologia 40:A161(abst), 1997
22) Kim KA, Kang K, Chi YI, Chang I, Lee MK, Kim KW, Shin SY, Lee MS. Identification and functional characterization of a novel mutation of hepatocyte nuclear factor-1 alpha gene in a Korean family with MODY3, Diabetologia 46:721-727, 2003
23) Hwang JS, Shin CH, Yang SW, Jung SY, Huh N. Genetic and clinical characteristics of Korean maturity-onset diabetes of the young (MODY) patients, Diabetes Res Clin Pract 74:75-81, 2006