GCKR mutations in Japanese families with clustered type 2 diabetes

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Running Title: *GCKR* mutations in Japanese families

Abstract:

Objective

The aim was to investigate the genetic background of familial clustering of type 2 diabetes.

Subjects and Methods

We recruited Japanese families with a 3-generation history of diabetes. Genome-wide linkage analysis was performed assuming an autosomal dominant model. Genes in the linkage region were computationally prioritized using Endeavour. We sequenced the candidate genes, and the frequencies of detected nucleotide changes were then examined in normoglycemic controls.

Results

To exclude known genetic factors, we sequenced 6 maturity onset diabetes of the young (MODY) genes in 10 familial cases. Because we detected a MODY3 mutation *HNF1A* R583G in one case, we excluded this case from further investigation. Linkage analysis revealed a significant linkage region on 2p25-22 (LOD score = 3.47) for 4 families. The 23.6-Mb linkage region contained 106 genes. Those genes were scored by computational prioritization. Eleven genes, i.e., top 10% of 106 genes, were selected and considered them as primary candidates. Considering their functions, we eliminated 3 well characterized genes and finally sequenced 8 genes. *GCKR* ranked highly in the computational prioritization. Mutations (minor allele frequency less than 1%) in exons and the promoter of *GCKR* were found in index cases of the families (3 of 18 alleles) more frequently than in controls (0 of 36 alleles, P=0.033). In one pedigree with 9 affected members, the mutation *GCKR* g.6859C>G was concordant with affection status. No mutation in other 7 genes that ranked highly in the prioritization was concordant with affection status in families.

Conclusions

We propose that *GCKR* is a susceptibility gene in Japanese families with clustered diabetes. The family based approach seems to be complementary with a large population study.

Keywords: Genetic susceptibility, Linkage analysis, MODY, *HNF1A*, *GCKR*

Abbreviations:

GAD: Glutamic acid decarboxylase

GCKR: Glucokinase regulator

HLOD: Heterogeneity logarithm of the odds

HNF4α: Hepatocyte Nuclear Factor 4α

LOD: Logarithm of the odds

MAF: Minor allele frequency

MODY: Maturity onset diabetes of the young

RFLP: Restriction fragment length polymorphism

SNP: Single nucleotide polymorphism

1. Introduction

The national survey in 2007 reported that 8.9 million people suffer from diabetes in Japan [1]. Most of these have type 2 diabetes, and the number of such patients has increased continuously. Both genetic and environmental factors play important roles in the pathogenesis of type 2 diabetes [2].

To elucidate the genetic factors underlying the pathogenesis of type 2 diabetes in the Japanese population, several genome-wide linkage analyses in Japanese sib-pairs have been performed [3-5]. Linkage to 11p13–p12 is consistently implicated in these studies [5]. Recent successes with genome-wide association analyses in the Japanese population have revealed a susceptibility variant in *KCNQ1* located at 11p15.5 [6, 7], a locus not far from the region suggested in linkage analyses. The association of susceptibility loci including *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8*, and *HHEX* with diabetes has been established in Caucasian populations and replicated in the Japanese population [8]. However, the loci identified in association studies have uniformly small effect sizes, and can explain only a small portion of the genetic background of diabetes in the Japanese population. Approaches other than sib-pair linkage analyses and association analyses may therefore be required to elucidate a greater aspect of the genetic background of type 2 diabetes.

In the present study, we used a family-based approach, because high degrees of familial clustering can raise the relative risk and provide better insight to novel loci of larger effect size [9]. Familial clustering of diabetes is well known, the typical example being MODY [10]. On the other hand, in most families in Japan, familial clustering cannot be attributed to mutations of the 6 known MODY genes [10], and genetic predisposition in such families has not been ascertained.

We recruited families having a 3-generation history of diabetes and performed genome-wide linkage analysis. We selected candidate genes in the linked chromosomal region and searched for rare and common nucleotide changes the genes in familial cases and unaffected controls.

2. Material and Methods

2.1. Families and Additional Index Cases

We recruited patients from collaborating hospitals in Japan who had diabetes with a 3-generation family history, which is suggestive of autosomal dominant mode of inheritance [11]. If ≥2 family members with diabetes were alive and donated DNA, the families were regarded as suitable subjects for the present study. Families including members with positive GAD (Glutamic Acid Decarboxylase) antibody were excluded from the study. Four families met these criteria and were included in the linkage analysis (Figure 1). Affected status of the participants was determined in two ways. First, if participants had been diagnosed with diabetes and treated with oral hypoglycemic agents or insulin injection, they were regarded as affected. Second, if participants had not been treated with oral hypoglycemic agents or insulin injection, they underwent HbA1c (Hemoglobin A_{1c}) measurement for screening of impaired glucose tolerance. The value for HbA1c is estimated as an NGSP (US National Glycohemoglobin Standardization Program) equivalent value (%) calculated by the formula HbA1c (%) = HbA1c (JDS, Japanese Diabetes Society) (%) + 0.4%, considering the relational expression of HbA1c (JDS)(%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) [12]. If their HbA1c levels were ≥6.0%, they were also regarded as affected. HbA1c ≥6.0% is the level defined as possible diabetes mellitus in the 2007 survey of the Ministry of Labor, Health and Welfare of Japan [1]. In addition to these subjects, 6 index cases from other families with a 3-generation history of diabetes were included in the study (Supplementary Figure 1). In these families, although we confirmed the affected status of some of the family members, DNA samples were available

only for the index cases but not for other family members. Together with the 4 index cases from the families included in the linkage analysis, a total of 10 unrelated cases with a 3-generation history of diabetes were available for DNA sequencing. The clinical features of family members and additional index cases are shown in Table 1.

2.2. Normoglycemic controls

An annual medical check-up program was performed in Nyukawa district of Takayama City, Japan. Nine-hundred ninety local residents (430 men, 560 women) were recruited in the program and consented to donate their DNA. From 2002 to 2007, participants underwent physical examination and blood tests including fasting plasma glucose and HbA1c every year. We selected normoglycemic controls from the participants in the cohort. Subjects defined as normoglycemic controls had the following characteristics: HbA1c <6.0% and fasting plasma glucose <5.5mmol/l during 5-year follow-up span, and age ≥55. The number of subjects that satisfied the definition was 206 (81 men, 125 women).

2.3. Genotyping Family Members

Genomic DNA was extracted from blood samples with a QIAamp DNA Blood Mini Kit (Qiagen Inc). PCR amplification from genomic DNA was performed with fluorescence-labeled (6-FAM, HEX, NED) and tailed primers. PCR primers to analyze microsatellite markers comprised an approximately 10cM human index map (ABI Prism Linkage Mapping Set Version 2.5: 382 markers for 22 autosomes), and other microsatellite fine markers were designed according to information from the UniSTS map. PCR reactions

were carried out in 7.5 μl with 50 ng genomic DNA, using AmpliTaq Gold DNA Polymerase (Applied Biosystems) in a 2-step amplification program. DNA fragments were analyzed on an Applied Biosystems 3130 Genetic Analyzer. Genotyping errors and inconsistent relationships were checked with the use of GENEHUNTER (version 2.1) software [13]. If the results of genotyping were missed or ambiguous, we treated them as an unknown genotype in the linkage analysis. The rate of genotyping failure was 0.057% (7/11842).

2.4. Linkage and Haplotype Analyses

Both affected and unaffected family members were included in the linkage analysis. Participants with HbA1c level <6.0% were considered as unaffected if the age was ≥55 and as unknown if the age was <55, considering the assumed age-dependent penetrance of diabetes. The purpose of including members assigned as unknown was to increase the accuracy of haplotype estimation in affected members, although inclusion did not increase the statistical power. Multipoint parametric analyses for autosomes were run using GENEHUNTER assuming an autosomal dominant model [13]. Because locus heterogeneity could be associated with diabetes, LOD (log of the odds) score and HLOD (heterogeneity LOD) score were calculated. The disease allele frequency was set at 0.00001 and a phenocopy frequency of 0.00001 was assumed. Population allele frequencies for each microsatellite marker were assigned equal portions for individual alleles. We used a 2-stage design: first, all chromosomal regions were screened by genotyping at an approximately 10cM density (screening), and the regions where LOD scores were highest were considered potentially interesting. Second, these regions were further finely mapped at approximately 1- to 2-cM densities (fine mapping). Regions where LOD scores were above 3.3, a level

corresponding to genome-wide significance [9], were considered as linkage regions. Haplotypes were constructed with the GENEHUNTER program.

2.5. Prioritization of Candidate Genes

The 23.6-Mb linkage region on chromosome 2p25-22 contained 106 genes annotated in Ensemble genome browser (www.ensembl.org). The genes were computationally prioritized using Endeavour (www.esat.kuleuven.be/endeavour/) [14]. We selected 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B,* and *NEUROD1*) as training genes because a dominant mode of inheritance was assumed in the highly clustered families in linkage analysis. We adopted all databases available in Endeavour, which prioritized glucokinase regulator (*GCKR*) at the first rank.

2.6. Sequencing

We directly sequenced the coding exons of 6 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B,* and *NEUROD1*) in the 10 index cases. We sequenced *GCKR* including all exons found in the National Center for Biotechnology Information (NCBI) Evidence Viewer (www.ncbi.nlm.nih.gov) and the 2-kb promoter region in the index cases from families and in control subjects. We also selected other 7 genes that are highly prioritized within the 11th rank (10.3%) in the linkage region using Endeavour excluding 3 genes with known metabolic functions unrelated to glucose metabolism (Supplementary Table 1). We sequenced the entire coding exons of the 7 genes in the index cases from families included in the linkage analysis. Forward and reverse PCR primers for each exon were selected in an intronic sequence 50 bp

away from the intron/exon boundaries and primers to amplify the *GCKR* promoter region were also selected. Sequencing primer data for *GCKR* is shown in Supplementary Table 2. PCR products were run on 2% agarose gel, and the appropriate bands were excised and then purified with the use of the QIAquick Gel Extraction Kit (Qiagen). Sequencing results were analyzed on an ABI Prism 3130 Avant DNA sequencer (Applied Biosystems). Any nucleotide changes identified in sequencing were searched for SNPs (single nucleotide polymorphisms) in the dbSNP database (www.ncbi.nlm.nih.gov/SNP/).

2.7. Genotyping SNPs

If minor allele frequencies (MAF) of nucleotide changes identified in sequencing were unregistered in the HapMap JPT database on dbSNP as of April 2010 and the minor allele appeared in <2 of all subjects, MAF was determined in the expanded population. We defined mutation as MAF<1% [15]. To determine whether each nucleotide change was a mutation or not, we genotyped 105 normoglycemic controls randomly selected from the cohort (Supplementary Table 3), because genotyping of 210 normal chromosomes is necessary to achieve 80% power to detect a polymorphism present in 1% of the population [16]. The PCR-RFLP (restriction fragment length polymorphism) method for *HNF1A* R583G, *GCKR* g.-689G>A, *GCKR* g.-299G>A, *GCKR* E252K and *FOSL2* R198H and Taqman method for *GCKR* g.6859C>G were used.

2.8. Statistical analysis

Frequencies of mutations (MAF<1%) and common nucleotide changes (MAF≥1%)

identified in *GCKR* sequencing in the index cases and in normoglycemic controls were compared by the Fisher exact test with SAS software (version 8.2).

2.9. Ethics

The methods used in this study were approved by the Ethics Committee of the Kyoto University Institutional Review Board, and approved written informed consent was obtained from each participant.

3. Results

3.1. Characteristics of Family Members

Four families with a 3-generation history of diabetes were enrolled in this study (Figure 1, Table 1). Every family included no less than 1 member that had been diagnosed with diabetes before the age of 50. Sixteen members (6 men, 10 women) had previously been diagnosed with diabetes. Thirteen out of the 16 members with diabetes were lean (BMI<25). Six members were treated with insulin and another 10 members were treated with oral hypoglycemic agents. Twelve family members who had not been diagnosed with diabetes underwent HbA1c measurement and 3 of them had HbA1c level ≥ 6.0%. These 3 members had already been diagnosed with impaired glucose tolerance before this study and were included as affected members in the study.

3.2. Exclusion of MODY gene mutations in the index cases

For the 10 index cases, we performed direct sequencing in entire coding exons of the MODY genes. The detected missense SNPs were *HNF1A* I27L (rs1169288), *HNF1A* S487N (rs2464196), *HNF1A* R583G, and *HNF4A* T117I (rs1800961) (Supplementary Table 4). *HNF1A* R583G is a mutation that is reported to cause MODY [17], thus we excluded the carrier of the mutation (additional index case #6, Table 1) from further investigation. *HNF1A* I27L and *HNF1A* S487N are common in the general population (MAF=0.386 and 0.341, respectively in HapMap-JPT). *HNF4A* T117I was associated with late-onset type 2 diabetes but it was not the cause of MODY in a previous report [18].

3.3. Linkage Analysis

A total of 30 members (19 affected members) from 4 families were included in the linkage analysis, assuming an autosomal dominant model. The genome-wide linkage results in the screening are shown in Figure 2. Regions of potential interest by multipoint LOD and HLOD scores were observed on chromosomes 2p24 and 7q34. After fine mapping, 2p25-22 was revealed to be a significant linkage region (Figure 3, LOD and HLOD=3.47) while the region on 7q34 was discarded. The size of the region with positive HLOD score was 23.6Mb (D2S2199-D2S2230). In the region, a haplotype segregated in affected and unaffected members in the pedigrees 1, 2, and 3, but not in the pedigree 4.

3.4. Candidate Genes

We searched candidate genes in the implicated linkage region by applying a gene prioritization approach implemented in Endeavour software. We selected 6 MODY genes as training genes. The 2 top-ranked genes were glucokinase regulatory protein (*GCKR*) and nuclear receptor coactivator 1 (*NCOA1*). *GCKR* ranked high in prioritization using gene-gene interaction databases (first rank in 5 out of 7 interaction databases), mainly because the interaction of glucokinase and glucokinase regulatory protein has been demonstrated in previous studies [19, 20]. *NCOA1* also ranked high in prioritization using gene-gene interaction databases (second rank in 2 out of 7 interaction databases), because nuclear receptor coactivator 1 has been reported to interact with HNF4α (Hepatocyte Nuclear Factor 4α) as a coactivator [21]. Together with *GCKR* and *NCOA1*, genes that are highly prioritized

within the 11th rank (10.3% of annotated genes) were considered as candidate genes except 3 genes with well-characterized metabolic functions unrelated to glucose metabolism (Supplementary Table 1).

3.5. Direct Sequencing in GCKR and other candidate genes

We performed direct sequencing in exons and the 2-kb promoter region of *GCKR*. Sequencing was performed in 9 index cases from families and in 18 normoglycemic controls in parallel. The 18 control subjects were randomly selected from 206 normoglycemic controls (Supplementary Table 3). Detected sequence changes in the 9 index cases and 18 controls are shown in Table 2. Five nucleotide changes (g.-959 Insertion AATGTTG, E66E, E77G, g.9709G>A, and L446P) were considered to be common variants, because the minor allele was found in not less than 2 subjects out of a total of 27 case and control subjects. To determine whether or not each of the other nucleotide changes (g.-689G>A, g.-299G>A, E252K and g.6859C>G) was a mutation (MAF<1%), genotyping was performed in a total of 105 normoglycemic controls. g.-689G>A, g.-299G>A and g.6859C>G were not detected in the 105 controls, and were regarded as mutations, while E252K was detected in 4 controls out of 105 (MAF=1.9%) and was regarded as a common change. The number of alleles having mutations was thus significantly larger in the index cases from families than in the controls (3/18 alleles vs. 0/36 alleles, P=0.033, Fisher exact test).

We performed direct sequencing in the entire coding exons of other 7 candidate genes in index cases from 4 families. One misssense mutation *FOSL2* R198H (MAF=0.004 in normoglycemic controls) was detected. No other mutations were detected in other 6 genes (Supplementary Table 5).

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295 *3.6. Segregation of the mutations with the Phenotype in Pedigrees*

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297 In index cases from the 4 families included in the linkage analysis, 3 sequence changes of
298 *GCKR* were detected (g.-959 Insertion AATGTTG, g.6859C>G and L446P). We tested the
299 segregation of *GCKR* g.6859C>G, a mutation detected in pedigree 3, with the phenotype in
300 the pedigree. Another 2 changes (*GCKR* g.-959 Insertion AATGTG and *GCKR* L446P) were
301 commonly detected in controls (3/36 alleles and 11/36 alleles respectively). In pedigree 3,
302 *GCKR* g.6859C>G was detected in all 9 affected members, but was not detected in the
303 unaffected member (II-7). We performed linkage analysis and haplotype construction in
304 2p25-22 using the *GCKR* g.6859 genotype together with the microsatellite markers. The
305 parametric multipoint LOD score for pedigree 3 was 2.67 at the *GCKR* g.6859 locus.
306 Haplotype analysis revealed that all affected individuals in pedigree 3 shared a disease
307 haplotype within D2S2199-D2S2230, which includes *GCKR* g.6859G (Figure 4). In pedigree
308 3, another sequence change, *GCKR* L446P, was detected, but *GCKR* L446P did not
309 co-segregate with the disease. Haplotype analysis revealed that the minor allele of *GCKR*
310 L446P (g.11169C) resided on a different haplotype than *GCKR* g.6859G in affected subjects
311 III-11, 12, 13, 14 (Figure 4).

312 We tested the segregation of *FOSL2* R198H, a mutation detected in pedigree 4, with the
313 phenotype. *FOSL2* R198H was detected in 2 affected subjects (II-2, II-22) but not detected in
314 one subject (II-1).

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4. Discussion and Conclusions

Recent progress in genome-wide association studies has identified tens of type 2 diabetes susceptibility genes. Even so, only a small portion of the genetic background of diabetes has been explained in the Japanese population. The loci identified in association studies have only very small effect sizes. We hypothesized that rare disease variants with larger effect sizes remain to be discovered that may explain a greater part of the genetic background. Family-based linkage study is an important alternative for the identification of rare disease variants. Indeed, studies with large families with highly clustered diabetes have revealed important mutations involved in MODY and other dominantly inherited diabetes, including a *KCNJ11* mutation [22]. We therefore recruited families with a 3-generation history of diabetes. The validity of our strategy was strengthened by the fact that one case out of the 10 index cases recruited in our study carried a previously reported rare disease variant *HNF1A* R583G.

Our family analysis revealed a significant linkage region on chromosome 2p25-22 that has not been reported in previous Japanese sib-pair analyses [3-5]. Because our approach was based on a higher degree of familial clustering than sib-pair analyses, the linkage region suggested in the present study might well go undetected in sib-pair analyses that include an admixture of sib-pairs with both low and high degrees of familial clustering. In the present study, we conducted a computational approach targeting the linkage region on chromosome 2p25-22. One hundred and six known genes were present in this linkage region. Prioritization of the candidate gene was possible by integrating the information available from multiple publicly available databases [14]. *GCKR* and other 7 gens ranked high in the prioritization, and were selected as candidate genes.

GCKR regulates glucokinase (GCK), the first glycolytic enzyme, in liver. *GCKR*-null mice exhibit elevated postprandial glucose [19]. Adenoviral-mediated overexpression of *GCKR* in mouse liver increases GCK activity and lowers fasting blood glucose. It was suggested that GCKR, a competitive inhibitor of GCK activity, also has a paradoxical role in extending GCK half-life by stabilizing the enzyme [20]. If so, diminished expression of GCKR in human might cause decreased GCK activity in liver and lead to impaired liver glucose uptake, which suggests the *GCKR* mutation as a possible cause of the disease in linked families.

We sequenced entire exons and the 2-kb promoter region of *GCKR* in 9 index 3-generation cases and in 18 control subjects. The rare variants were significantly more frequent in index cases from families than in control subjects. In addition, exonic rare variant g.6859C>G in pedigree 3, which was not detected in 105 control subjects, was clearly segregated in all 9 affected members in pedigree 3. Previous reports have shown the association of common *GCKR* variants with fasting plasma glucose, glucose level after glucose challenge, and diabetes risk in various ethnic groups [23-30]. In Japanese population, a common variant *GCKR* rs780094 is associated with fasting glucose and diabetes risk [27, 30]. Our family study suggests the effect of rare *GCKR* variants on diabetes susceptibility that has not been revealed by previous association studies. A recent study has shown the excess of rare *GCKR* variants in individuals with hypertriglyceridemia [31], which supports our idea that rare *GCKR* mutations also affect the diabetes susceptibility.

On the other hand, the only one mutation in other 7 highly prioritized genes was *FOSL2* R198H and it did not co-segregate with the phenotype in the pedigree. Therefore, we tentatively eliminate the possibility that these genes are involved in familial clustering of diabetes patients in the current pedigrees.

Our study has several limitations. First is the large size (23.6Mb) of the linkage region.

Only 4 families could be included in the linkage analysis because we limited the cohort to 3-generation families with ≥2 affected members who donated DNA. Further efforts to recruit large families are needed to narrow down the linkage region. Second, because the *GCKR* g.6859C>G mutation was in a non-coding exon, confirming the relevance of the mutation as the cause of the disease is difficult. Investigation of the effect of the mutation in human liver, where GCKR is predominantly expressed [32], is required, but liver specimens of family members are currently unavailable. Although we tried to determine the mRNA level in peripheral blood of family members, GCKR mRNA was only barely detectable with the RT-PCR method (data not shown), so comparison of the GCKR mRNA level between affected and unaffected members was not possible. We speculate that the g.6859C>G mutatin might affect GCKR function in liver through mRNA transcription or splicing processes [33]. *GCKR* g.-689G>A and g.-299G>A mutations located in the promoter also might affect the expression of GCKR, but TRANSFAC database [34] expected no binding sites of transcription factors at the two promoter mutations.

In conclusion, with systematic investigation we propose that *GCKR* is a susceptibility gene in Japanese families with clustered diabetes. A family-based approach may be a promising strategy to elucidate the complex genetic background of common diseases including type 2 diabetes.

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591 Table 1. Characteristics of family members and additional index cases.

	ID	Current Age	Sex	BMI	HbA1c(%)	Age when diagnosed (Diagnosis)	Current therapy
Pedigree 1	II-4	70	F	16.2	5.0		
	II-5	71	F	22.5	10.6	60 (DM)	Insulin 66U/d
	III-1	40	F	21.9	5.4		
	III-2	37	M	26.0	6.9	20 (DM)	Insulin
Pedigree 2	II-1	79	M	19.2	7.5	50 (DM)	Insulin 25U/d
	II-2	77	F	18.6	5.6		
	II-3	76	M	17.9	7.2	45 (DM)	Insulin
	II-5	74	M	18.2	6.0	64 (IGT)	Diet
	II-6	71	F	18.4	6.6	N/A (DM)	Oral drug
	II-7	68	F	19.9	5.9		
	III-1	53	M	24.2	6.0	53 (IGT)	Diet
	III-3	51	M	20.4	5.6		
	III-4	47	F	19.3	5.2		
	III-5	46	F	19.6	4.9		
	IV-1	23	M	19.9	5.6		
Pedigree 3	II-7	92	F	22.3	5.9		
	III-2	77	F	23.9	9.3	30 (DM)	Oral drug
	III-5	72	F	22.0	8.1	60 (DM)	Insulin 16U/d
	III-6	69	F	19.8	8.0	65 (DM)	Insulin 16U/d
	III-8	66	F	19.1	6.5	64 (IGT)	Diet
	III-10	59	F	19.3	10.2	57 (DM)	Oral drug
	III-11	67	F	20.4	6.9	62 (DM)	Oral drug
	III-12	66	M	21.1	N/A	57 (DM)	Oral drug
	III-13	64	F	20.0	6.6	25 (DM)	Insulin
	III-14	62	M	20.2	10.3	50 (DM)	Oral drug
Pedigree 4	II-1	76	F	28.2	6.7	60 (DM)	Oral drug
	II-2	73	F	25.1	6.4	50 (DM)	Oral drug
	II-3	67	F	19.0	5.5		
	II-4	64	M	N/A	5.4		
	III-1	52	F	20.4	5.3		
	III-2	50	M	20.8	6.2	35 (DM)	Oral drug
Additional Index Cases	1	57	M	25.7	7.1	30 (DM)	Oral drug
	2	47	F	22.9	10.0	36 (DM)	Insulin 20U/d
	3	68	F	19.7	7.1	45 (DM)	Insulin 19U/d
	4	60	F	24.7	10.4	40 (DM)	Insulin 51U/d
	5	60	F	28.0	9.7	50 (DM)	Insulin 8U/d
	6	54	F	34.5	9.1	40 (DM)	Insulin

BMI: Body Mass Index, DM: Diabetes Mellitus, IGT: Impaired Glucose Toleance

Table 2. Mutations and common nucleotide changes in exons and the promoter of *GCKR* in 9 index cases in families and in 18 controls.

Position	Change	Description	Effect	Detected Number of Alleles: Index Cases from Families (n=9) Major	Index Cases from Families (n=9) Minor	Controls(n=18) Major	Controls(n=18) Minor	*p* [a]	Minor Allele Frequency [MAF]
Mutations (MAF<1%)									
Promoter	g. -689G>A			17	1	36	0	0.33	0.000 [b]
Promoter	g. -299G>A			17	1	36	0	0.33	0.000 [b]
Exon 9	g. 6859C>G	Noncoding exon		17	1	36	0	0.33	0.000 [b]
Total				15	3	36	0	0.033	
Common changes									
Promoter	g.-959 insAATGTTG			16	2	33	3	1.00	N/D
Exon 2	g. 468G>A	Synonymous	E66E	17	1	35	1	1.00	N/D
Exon 3	g. 671A>G	Missense	E77G	17	1	33	3	1.00	0.024 [c]
Exon 10	g. 8817G>A	Missense	E252K	18	0	35	1	1.00	0.019 [b]
Exon 11	g. 9709G>A	Noncoding exon		17	1	33	3	1.00	0.123 [c]
Exon 14	g. 11169T>C	Missense	L446P	8	10	25	11	0.087	0.467 [c]

GenBank Accession No. NT_022184.15

[a] Fisher exact test. [b]Frequency in 105 normoglycemic controls. [c]Frequency in HapMap-JPT.

Figure Captions

Figure 1. Four pedigrees with familial aggregated diabetes mellitus.

Figure 2. Multipoint HLOD and LOD scores in genome-wide linkage analysis for 4 pedigrees.

Figure 3. Multipoint HLOD and LOD scores in fine mapping of D2S168-D2S2259 and D7S640-D7S636.

Figure 4. Haplotype analysis in the D2S168-D2S2259 region and the *GCKR* g. 6859C>G genotype for pedigree 3.

Figure 1.

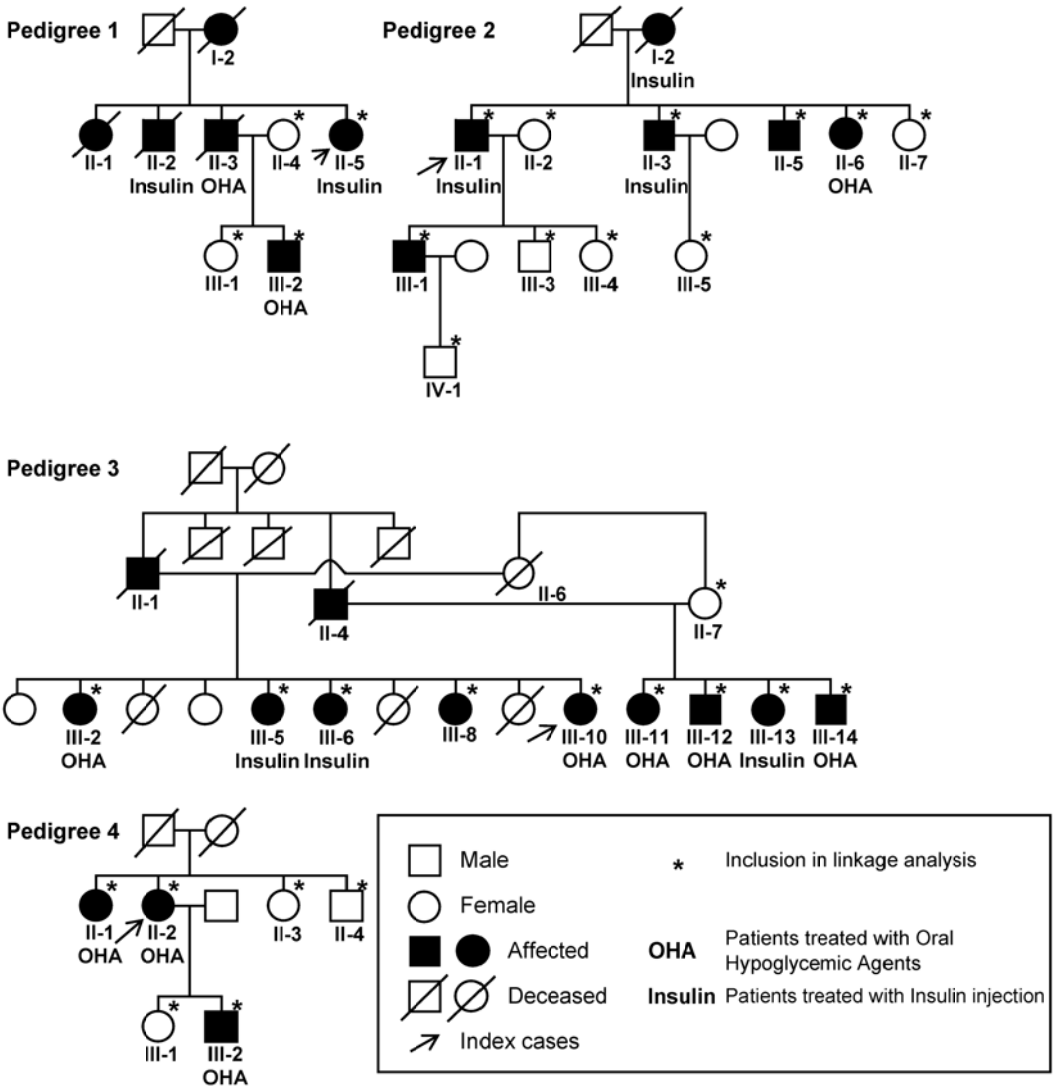

Pedigree 1
I-2
II-1
II-2
Insulin
II-3
OHA
II-4
II-5
Insulin
III-1
III-2
OHA
Pedigree 2
I-2
Insulin
II-1
Insulin
II-2
II-3
Insulin
II-5
II-6
OHA
II-7
III-1
III-3
III-4
III-5
IV-1
Pedigree 3
II-1
II-4
II-6
II-7
III-2
OHA
III-5
Insulin
III-6
Insulin
III-8
III-10
OHA
III-11
OHA
III-12
OHA
III-13
Insulin
III-14
OHA
Pedigree 4
II-1
OHA
II-2
OHA
II-3
II-4
III-1
III-2
OHA
Male
Female
Affected
Deceased
Index cases
Inclusion in linkage analysis
OHA
Patients treated with Oral Hypoglycemic Agents
Insulin
Patients treated with Insulin injection

Figure 2.

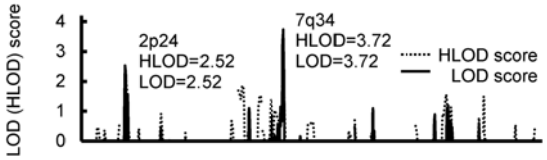

LOD (HLOD) score
4
3
2
1
0
2p24
HLOD=2.52
LOD=2.52
7q34
HLOD=3.72
LOD=3.72
HLOD score
LOD score

Figure 3.

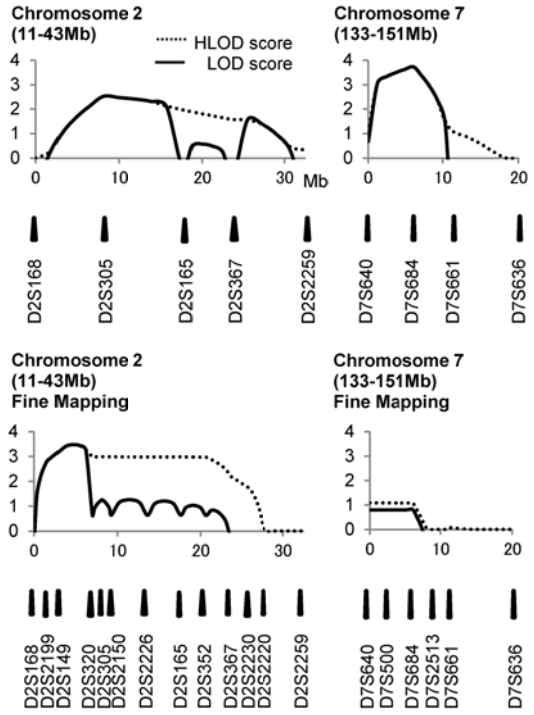
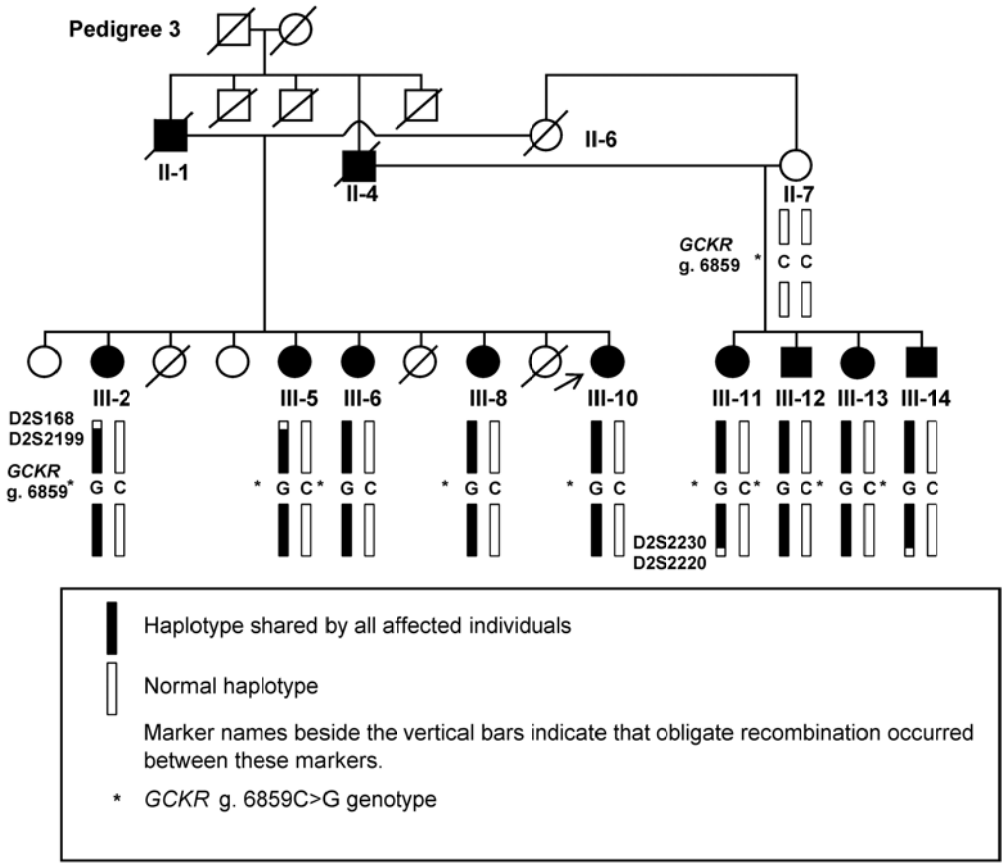

Chromosome 2
(11-43Mb)
HLOD score
LOD score
Mb
D2S168
D2S305
D2S165
D2S367
D2S2259
Chromosome 7
(133-151Mb)
D7S640
D7S684
D7S661
D7S636
Chromosome 2
(11-43Mb)
Fine Mapping
D2S168
D2S2199
D2S149
D2S320
D2S305
D2S2150
D2S2226
D2S165
D2S352
D2S367
D2S2230
D2S2220
D2S2259
Chromosome 7
(133-151Mb)
Fine Mapping
D7S640
D7S500
D7S684
D7S2513
D7S661
D7S636

Figure 4.


Pedigree 3
II-1
II-4
II-6
II-7
GCKR g. 6859 * C C
III-2
III-5
III-6
III-8
III-10
III-11
III-12
III-13
III-14
D2S168
D2S2199
GCKR g. 6859*
G C
D2S2230
D2S2220
Haplotype shared by all affected individuals
Normal haplotype
Marker names beside the vertical bars indicate that obligate recombination occurred between these markers.
* GCKR g. 6859C>G genotype

Supplementary Table 1. Genes that are highly prioritized in the linkage region using Endeavour.

Rank	Symbol	Description	Known metabolic function
1	GCKR	Glucokinase regulatory protein	
2	NCOA1	Nuclear receptor coactivator 1	
3	FOSL2	Fos-related antigen 2	
4	APOB	Apolipoprotein B-100 precursor	Lipid Metabolism [a]
5	MYCN	N-myc proto-oncogene protein	
6	RBKS	Ribokinase	
7	XDH	Xanthine dehydrogenase/oxidase	Purine Metabolism [a]
8	KHK	Ketohexokinase	
9	NRBP1	Nuclear receptor-binding protein	
10	CAD	Glutamine-dependent carbamoyl-phosphate synthase, Aspartate carbamoyltransferase, Dihydroorotase	Pyrimidine Metabolism [a]
11	RDH14	Retinol dehydrogenase	

[a] Excluded from candidate genes

Supplementary Table 2. Sequencing Primers for *GCKR*

GCKR

	Forward		Reverse
promoter1a	TGACTAGCTGTGGTTGACCCT	promoter1b	ATCTCCCTCACTCTCTCCCCT
promoter2a	CCCCATCCCTTATCCCTTCT	promoter2b	TGGACAAATTGGGACTCACA
promoter3a	GGGTTGTTGTGAGGCTCAAAT	promoter3b	TCCGGGGTCTCAATGACAT
1a	TAGTGACCAGGAAAGGGTGGT	1b	CCAAAAGGGAGAAAGGAGAA
1c	TAATATGCCCAGAGCACCAA		
2a	AGCAAGACATGGGAGTCAAA	2b	TGAGGGAATAAGGAATGGTGA
3a	AATGTAGCCTGCCCTAATACG	3b	CCTTCTAGCACCGATCTCATT
4a	TTCTGATGCACTTGAGCCTT	4b	TTATAAGCTTAGGGGCACCC
5a	ACCTCAATCCCAATGCAGTCT	5b	TAATCCCAGCTACTCCGCAGA
5c	AGAGCGTTGAATAGCCATTG		
6a	TGGTACTATCACATGCATGCC	6b	TGGTGGGCTGCAGTCTTACT
7a	TAAGGGAGCTGTGCCTTCA	7b	TTCCAATGAACTTCCCACCT
		7c	TTAGATAGGGAAGGTGGGACA
8a	AGTGTTAGATCTCCTCCACGG	8b	AGGGTCAGAGAGGTCTCCAAA
9a	ATTTAAACGCTGGGCTGCT	9b	AGAAGCACACAGAAAAGGCA
10a	ATCCCAGCCTCTCACTCTCAT	10b	CCACTGAGCTTTGTAAACCCA
11a	TGAACTTAAGTGATCTGCCCA	11b	AGGGATGCCAGTATAAGGCTT

Supplementary Table 3. Characteristics of normoglycemic controls.

		Subjects for sequencing (n=18)	Subjects for genotyping SNPs (n=105)
Men	Number	10	52
	Age (y: mean ± SD)	64.0 ± 4.4	68.3 ± 8.1
	BMI (mean ± SD)	22.6 ± 2.0	22.5 ± 2.6
	Fasting Plasma Glucose (mmol/l: mean± SD)	4.86 ± 0.21	4.74 ± 0.31
	HbA1c (%: mean±SD)	5.64 ± 0.12	5.45± 0.17
Women	Number	8	53
	Age (y: mean ± SD)	61.1 ± 6.1	66.5 ± 8.3
	BMI (mean ± SD)	21.4 ± 2.3	21.7 ± 2.8
	Fasting Plasma Glucose (mmol/l: mean± SD)	4.66 ± 0.29	4.68 ± 0.38
	HbA1c (%: mean ± SD)	5.64 ± 0.20	5.48 ± 0.16

SD: Standard deviation.

Supplementary Table 4. Missense SNPs of MODY genes in 10 index cases.

Gene	SNP	Major/Minor	Number of Alleles in Index cases (n=10)		Minor Allele Frequency [MAF]
			Major	Minor	
HNF1A	I27L	C/A	8	12	0.386 [c]
HNF1A	S487N	A/G	9	11	0.341 [c]
HNF1A	R583G	C/G	19	1[a]	0.000 [d]
HNF4A	T117I	C/T	19	1[b]	0.000 [c]

[a] Additional index case #6.
[b] Additional index case #2.
[c] Frequency in HapMap-JPT.
[d] Frequency in 105 normoglycemic controls.

Supplementary Table 5. Nucleotide changes in coding exons of 7 genes in the linkage region in index cases from 4 families.

Gene	SNP	Major/Minor	Number of Alleles in Index cases (n=4)		Minor Allele Frequency [MAF]
			Major	Minor	
NCOA1	P504P	A/T	7	1	0.307 [a]
FOSL2	R198H	G/A	7	1	0.004 [b]
KHK	V49I	G/A	5	3	0.166 [a]

[a] Frequency in HapMap-JPT.
[b] Frequency in 105 normoglycemic controls.

Supplementary Figure 1. Pedigrees of Additional Index Cases