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Author(s)	Tanaka, Daisuke; Nagashima, Kazuaki; Sasaki, Mayumi; Yamada, Chizumi; Funakoshi, Shogo; Akitomo, Kimiyo; Takenaka, Katsunobu; Harada, Kouji; Koizumi, Akio; Inagaki, Nobuya
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1 **GCKR mutations in Japanese families with clustered type 2 diabetes**

2

3 Daisuke Tanaka^a, Kazuaki Nagashima^a, Mayumi Sasaki^a, Chizumi Yamada^a, Shogo
4 Funakoshi^a, Kimiyo Akitomo^a, Katsunobu Takenaka^b, Kouji Harada^c, Akio Koizumi^c, and
5 Nobuya Inagaki^a

6

7 ^a*Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto*
8 *University, Kyoto, Japan*

9 ^b*Takayama Red Cross Hospital, Gifu, Japan*

10 ^c*Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto*
11 *University, Kyoto, Japan*

12

13 **Corresponding Author:**

14 Nobuya Inagaki

15 Department of Diabetes and Clinical Nutrition,

16 Graduate School of Medicine, Kyoto University

17 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan

18 Telephone: +81-75-751-3562

19 Fax: +81-75-771-6601

20 E-mail: inagaki@metab.kuhp.kyoto-u.ac.jp

21

22 **Running Title:** GCKR mutations in Japanese families

23

24 **Abstract:**

25 **Objective**

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

27 **Subjects and Methods**

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

33 **Results**

34 To exclude known genetic factors, we sequenced 6 maturity onset diabetes of the young
35 (MODY) genes in 10 familial cases. Because we detected a MODY3 mutation *HNF1A*
36 R583G in one case, we excluded this case from further investigation. Linkage analysis
37 revealed a significant linkage region on 2p25-22 (LOD score = 3.47) for 4 families. The
38 23.6-Mb linkage region contained 106 genes. Those genes were scored by computational
39 prioritization. Eleven genes, i.e., top 10% of 106 genes, were selected and considered them as
40 primary candidates. Considering their functions, we eliminated 3 well characterized genes
41 and finally sequenced 8 genes. *GCKR* ranked highly in the computational prioritization.
42 Mutations (minor allele frequency less than 1%) in exons and the promoter of *GCKR* were
43 found in index cases of the families (3 of 18 alleles) more frequently than in controls (0 of 36
44 alleles, P=0.033). In one pedigree with 9 affected members, the mutation *GCKR* g.6859C>G
45 was concordant with affection status. No mutation in other 7 genes that ranked highly in the
46 prioritization was concordant with affection status in families.

47 **Conclusions**

48 We propose that *GCKR* is a susceptibility gene in Japanese families with clustered diabetes.

49 The family based approach seems to be complementary with a large population study.

50

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

52

53 **Abbreviations:**

54 GAD: Glutamic acid decarboxylase

55 GCKR: Glucokinase regulator

56 HLOD: Heterogeneity logarithm of the odds

57 HNF4 α : Hepatocyte Nuclear Factor 4 α

58 LOD: Logarithm of the odds

59 MAF: Minor allele frequency

60 MODY: Maturity onset diabetes of the young

61 RFLP: Restriction fragment length polymorphism

62 SNP: Single nucleotide polymorphism

63

64 **1. Introduction**

65

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

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

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

88 We recruited families having a 3-generation history of diabetes and performed
89 genome-wide linkage analysis. We selected candidate genes in the linked chromosomal
90 region and searched for rare and common nucleotide changes the genes in familial cases and
91 unaffected controls.
92

93 2. Material and Methods

94

95 2.1. Families and Additional Index Cases

96

97 We recruited patients from collaborating hospitals in Japan who had diabetes with a
98 3-generation family history, which is suggestive of autosomal dominant mode of inheritance
99 [11]. If ≥ 2 family members with diabetes were alive and donated DNA, the families were
100 regarded as suitable subjects for the present study. Families including members with positive
101 GAD (Glutamic Acid Decarboxylase) antibody were excluded from the study. Four families
102 met these criteria and were included in the linkage analysis (Figure 1). Affected status of the
103 participants was determined in two ways. First, if participants had been diagnosed with
104 diabetes and treated with oral hypoglycemic agents or insulin injection, they were regarded as
105 affected. Second, if participants had not been treated with oral hypoglycemic agents or insulin
106 injection, they underwent HbA1c (Hemoglobin A_{1c}) measurement for screening of impaired
107 glucose tolerance. The value for HbA1c is estimated as an NGSP (US National
108 Glycohemoglobin Standardization Program) equivalent value (%) calculated by the formula
109 $\text{HbA1c (\%)} = \text{HbA1c (JDS, Japanese Diabetes Society) (\%)} + 0.4\%$, considering the
110 relational expression of HbA1c (JDS)(%) measured by the previous Japanese standard
111 substance and measurement methods and HbA1c (NGSP) [12]. If their HbA1c levels were
112 $\geq 6.0\%$, they were also regarded as affected. HbA1c $\geq 6.0\%$ is the level defined as possible
113 diabetes mellitus in the 2007 survey of the Ministry of Labor, Health and Welfare of Japan [1].
114 In addition to these subjects, 6 index cases from other families with a 3-generation history of
115 diabetes were included in the study (Supplementary Figure 1). In these families, although we
116 confirmed the affected status of some of the family members, DNA samples were available

117 only for the index cases but not for other family members. Together with the 4 index cases
118 from the families included in the linkage analysis, a total of 10 unrelated cases with a
119 3-generation history of diabetes were available for DNA sequencing. The clinical features of
120 family members and additional index cases are shown in Table 1.

121

122 *2.2. Normoglycemic controls*

123

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

132

133 *2.3. Genotyping Family Members*

134

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

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

147

148 *2.4. Linkage and Haplotype Analyses*

149

150 Both affected and unaffected family members were included in the linkage analysis.
151 Participants with HbA1c level <6.0% were considered as unaffected if the age was ≥ 55 and
152 as unknown if the age was <55, considering the assumed age-dependent penetrance of
153 diabetes. The purpose of including members assigned as unknown was to increase the
154 accuracy of haplotype estimation in affected members, although inclusion did not increase the
155 statistical power. Multipoint parametric analyses for autosomes were run using
156 GENEHUNTER assuming an autosomal dominant model [13]. Because locus heterogeneity
157 could be associated with diabetes, LOD (log of the odds) score and HLOD (heterogeneity
158 LOD) score were calculated. The disease allele frequency was set at 0.00001 and a
159 phenocopy frequency of 0.00001 was assumed. Population allele frequencies for each
160 microsatellite marker were assigned equal portions for individual alleles. We used a 2-stage
161 design: first, all chromosomal regions were screened by genotyping at an approximately
162 10cM density (screening), and the regions where LOD scores were highest were considered
163 potentially interesting. Second, these regions were further finely mapped at approximately 1-
164 to 2-cM densities (fine mapping). Regions where LOD scores were above 3.3, a level

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

167

168 *2.5. Prioritization of Candidate Genes*

169

170 The 23.6-Mb linkage region on chromosome 2p25-22 contained 106 genes annotated in
171 Ensemble genome browser (www.ensembl.org). The genes were computationally prioritized
172 using Endeavour (www.esat.kuleuven.be/endeavour/) [14]. We selected 6 MODY genes
173 (*HNFB4A*, *GCK*, *HNFB1A*, *PDX1*, *HNFB1B*, and *NEUROD1*) as training genes because a
174 dominant mode of inheritance was assumed in the highly clustered families in linkage
175 analysis. We adopted all databases available in Endeavour, which prioritized glucokinase
176 regulator (*GCKR*) at the first rank.

177

178 *2.6. Sequencing*

179

180 We directly sequenced the coding exons of 6 MODY genes (*HNFB4A*, *GCK*, *HNFB1A*, *PDX1*,
181 *HNFB1B*, and *NEUROD1*) in the 10 index cases. We sequenced *GCKR* including all exons
182 found in the National Center for Biotechnology Information (NCBI) Evidence Viewer
183 (www.ncbi.nlm.nih.gov) and the 2-kb promoter region in the index cases from families and in
184 control subjects. We also selected other 7 genes that are highly prioritized within the 11th
185 rank (10.3%) in the linkage region using Endeavour excluding 3 genes with known metabolic
186 functions unrelated to glucose metabolism (Supplementary Table 1). We sequenced the entire
187 coding exons of the 7 genes in the index cases from families included in the linkage analysis.
188 Forward and reverse PCR primers for each exon were selected in an intronic sequence 50 bp

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

196

197 2.7. Genotyping SNPs

198

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

209

210 2.8. Statistical analysis

211

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

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

215

216 *2.9. Ethics*

217

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

221

222 3. Results

223

224 3.1. Characteristics of Family Members

225

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

235

236 3.2. Exclusion of MODY gene mutations in the index cases

237

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

246

247 3.3. Linkage Analysis

248

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

257

258 3.4. Candidate Genes

259

260 We searched candidate genes in the implicated linkage region by applying a gene
261 prioritization approach implemented in Endeavour software. We selected 6 MODY genes as
262 training genes. The 2 top-ranked genes were glucokinase regulatory protein (*GCKR*) and
263 nuclear receptor coactivator 1 (*NCOA1*). *GCKR* ranked high in prioritization using gene-gene
264 interaction databases (first rank in 5 out of 7 interaction databases), mainly because the
265 interaction of glucokinase and glucokinase regulatory protein has been demonstrated in
266 previous studies [19, 20]. *NCOA1* also ranked high in prioritization using gene-gene
267 interaction databases (second rank in 2 out of 7 interaction databases), because nuclear
268 receptor coactivator 1 has been reported to interact with HNF4 α (Hepatocyte Nuclear Factor
269 4 α) as a coactivator [21]. Together with *GCKR* and *NCOA1*, genes that are highly prioritized

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

273

274 3.5. Direct Sequencing in *GCKR* and other candidate genes

275

276 We performed direct sequencing in exons and the 2-kb promoter region of *GCKR*.
277 Sequencing was performed in 9 index cases from families and in 18 normoglycemic controls
278 in parallel. The 18 control subjects were randomly selected from 206 normoglycemic controls
279 (Supplementary Table 3). Detected sequence changes in the 9 index cases and 18 controls are
280 shown in Table 2. Five nucleotide changes (g.-959 Insertion AATGTTG, E66E, E77G,
281 g.9709G>A, and L446P) were considered to be common variants, because the minor allele
282 was found in not less than 2 subjects out of a total of 27 case and control subjects. To
283 determine whether or not each of the other nucleotide changes (g.-689G>A, g.-299G>A,
284 E252K and g.6859C>G) was a mutation (MAF<1%), genotyping was performed in a total of
285 105 normoglycemic controls. g.-689G>A, g.-299G>A and g.6859C>G were not detected in
286 the 105 controls, and were regarded as mutations, while E252K was detected in 4 controls out
287 of 105 (MAF=1.9%) and was regarded as a common change. The number of alleles having
288 mutations was thus significantly larger in the index cases from families than in the controls
289 (3/18 alleles vs. 0/36 alleles, P=0.033, Fisher exact test).

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

294

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

296

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 AATGTTG 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).

315

316 4. Discussion and Conclusions

317

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

330 Our family analysis revealed a significant linkage region on chromosome 2p25-22 that has
331 not been reported in previous Japanese sib-pair analyses [3-5]. Because our approach was
332 based on a higher degree of familial clustering than sib-pair analyses, the linkage region
333 suggested in the present study might well go undetected in sib-pair analyses that include an
334 admixture of sib-pairs with both low and high degrees of familial clustering. In the present
335 study, we conducted a computational approach targeting the linkage region on chromosome
336 2p25-22. One hundred and six known genes were present in this linkage region. Prioritization
337 of the candidate gene was possible by integrating the information available from multiple
338 publicly available databases [14]. *GCKR* and other 7 genes ranked high in the prioritization,
339 and were selected as candidate genes.

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

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

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

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

364 Only 4 families could be included in the linkage analysis because we limited the cohort to
365 3-generation families with ≥ 2 affected members who donated DNA. Further efforts to recruit
366 large families are needed to narrow down the linkage region. Second, because the *GCKR*
367 g.6859C>G mutation was in a non-coding exon, confirming the relevance of the mutation as
368 the cause of the disease is difficult. Investigation of the effect of the mutation in human liver,
369 where GCKR is predominantly expressed [32], is required, but liver specimens of family
370 members are currently unavailable. Although we tried to determine the mRNA level in
371 peripheral blood of family members, GCKR mRNA was only barely detectable with the
372 RT-PCR method (data not shown), so comparison of the GCKR mRNA level between
373 affected and unaffected members was not possible. We speculate that the g.6859C>G mutatin
374 might affect GCKR function in liver through mRNA transcription or splicing processes [33].
375 *GCKR* g.-689G>A and g.-299G>A mutations located in the promoter also might affect the
376 expression of GCKR, but TRANSFAC database [34] expected no binding sites of
377 transcription factors at the two promoter mutations.

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

382

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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			
Pedigree 3	IV-1	23	M	19.9	5.6			
	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 Tolerance

592 Table 2. Mutations and common nucleotide changes in exons and the promoter of *GCKR* in 9 index cases in families and in 18 controls.
 593

Position	Change	Description	Effect	Detected Number of Alleles				<i>p</i> ^a	Minor Allele Frequency [MAF]
				Index Cases from Families (n=9)		Controls(n=18)			
				Major	Minor	Major	Minor		
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

594 GenBank Accession No. NT_022184.15

595 ^a Fisher exact test. ^bFrequency in 105 normoglycemic controls. ^cFrequency in HapMap-JPT.

596

597 **Figure Captions**

598

599 **Figure 1.** Four pedigrees with familial aggregated diabetes mellitus.

600

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

602

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

604

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

606

Figure 1.

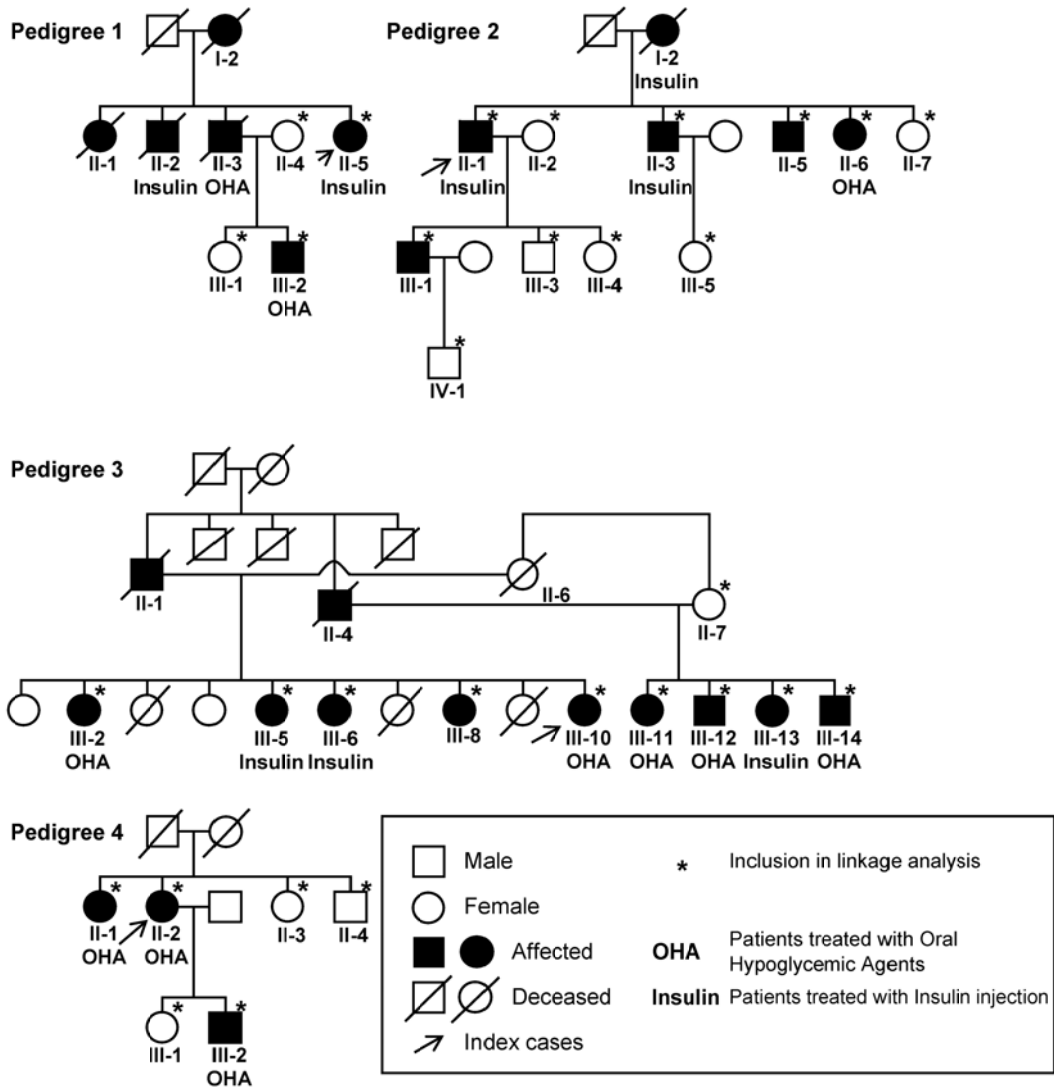


Figure 2.

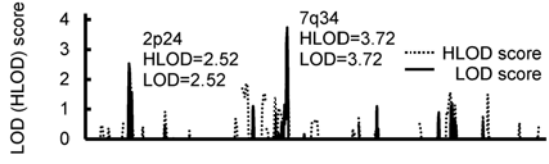


Figure 3.

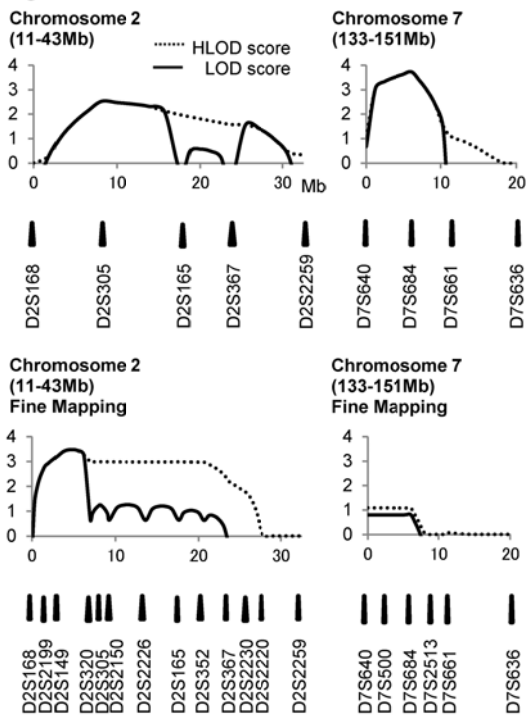
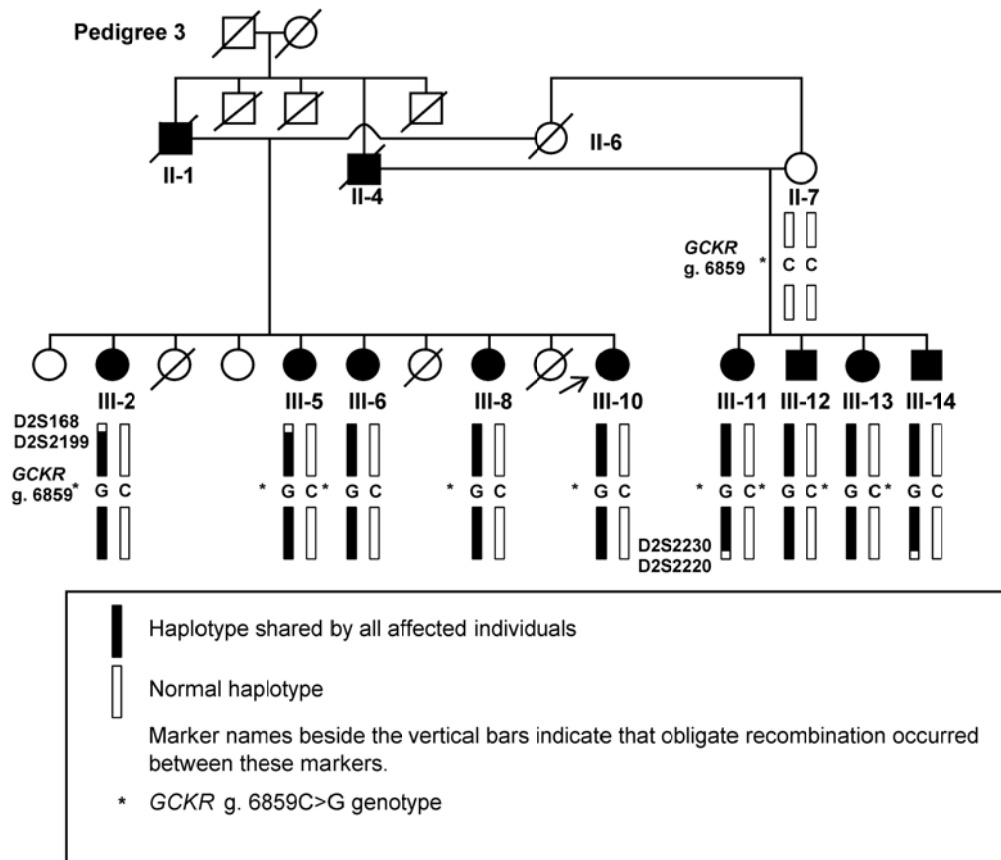


Figure 4.



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	TAATATGCCAGAGCACCAA		
2a	AGCAAGACATGGGAGTCAAA	2b	TGAGGGAATAAGGAATGGTGA
3a	AATGTAGCCTGCCCTAATACG	3b	CCTTCTAGCACCGATCTCATT
4a	TTCTGATGCACTTGAGCCTT	4b	TTATAAGCTTAGGGGCACCC
5a	ACCTCAATCCAATGCAGTCT	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 \pm SD)	64.0 \pm 4.4	68.3 \pm 8.1
	BMI (mean \pm SD)	22.6 \pm 2.0	22.5 \pm 2.6
	Fasting Plasma Glucose (mmol/l: mean \pm SD)	4.86 \pm 0.21	4.74 \pm 0.31
	HbA1c (%: mean \pm SD)	5.64 \pm 0.12	5.45 \pm 0.17
Women	Number	8	53
	Age (y: mean \pm SD)	61.1 \pm 6.1	66.5 \pm 8.3
	BMI (mean \pm SD)	21.4 \pm 2.3	21.7 \pm 2.8
	Fasting Plasma Glucose (mmol/l: mean \pm SD)	4.66 \pm 0.29	4.68 \pm 0.38
	HbA1c (%: mean \pm SD)	5.64 \pm 0.20	5.48 \pm 0.16

SD: Standard deviation.

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

Gene	SNP	Major/Minor	Number of Alleles		Minor Allele Frequency [MAF]
			Index cases (n=10)		
			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.

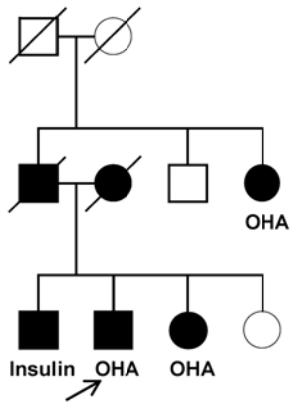
Gene	SNP	Major/Minor	Number of Alleles		Minor Allele Frequency [MAF]
			Index cases (n=4)		
			Major	Minor	
NCOA1	P504P	A/T	7	1	0.307 ^a
FOSL2	R198H	G/A	7	1	0.004 ^b
KHK	V49I	G/A	5	3	0.166 ^a

^a Frequency in HapMap-JPT.

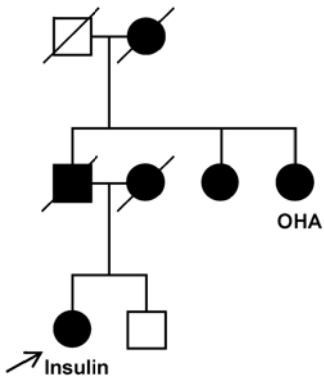
^b Frequency in 105 normoglycemic controls.

Supplementary Figure 1. Pedigrees of Additional Index Cases

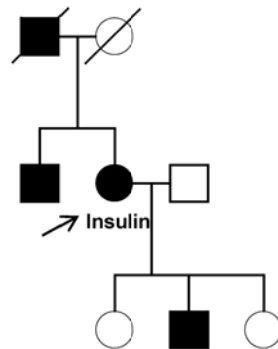
Index Case 1



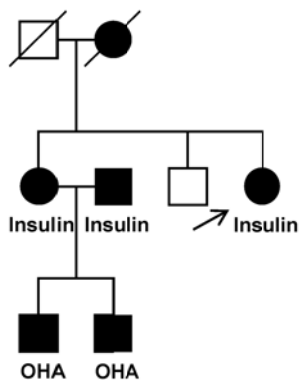
Index Case 2



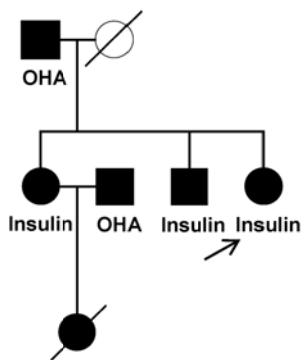
Index Case 3



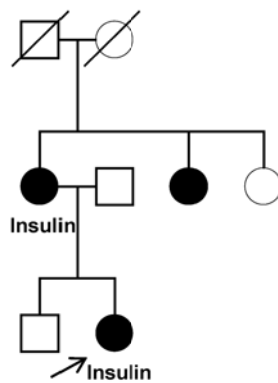
Index Case 4



Index Case 5



Index Case 6



OHA	Patients treated with Oral Hypoglycemic Agents
Insulin	Patients treated with Insulin injection