# Interaction and Association Analysis of a Type 1 Diabetes Susceptibility Locus on Chromosome 5q11-q13 and the 7q32 Chromosomal Region in Scandinavian Families

**Pernilla Holm,[1] Berit Rydlander,[1] Holger Luthman,[2] Ingrid Kockum,[1] for the European Consortium for IDDM Genome Studies**

**We have previously reported suggestive linkage to chromosome 5p13-q13 in type 1 diabetic families. ISL1, a transcription factor involved in pancreas development, maps to this region. Sequencing of the ISL1 gene in patients and control subjects identified seven single nucleotide polymorphisms (SNPs) and one microsatellite in noncoding regions. Four haplotypes formed by six of these SNPs and one microsatellite were associated with type 1 diabetes in Swedish families ($P < 0.04$). To identify possible interactions with the 5q11-q13 region, we applied pathway-restricted linkage analysis by analyzing for effects from regions encoding other transcription factors that are active during pancreas development and maintenance of insulin production. Linkage analysis allowing for interaction between 5q11-q13 and 7q32 resulted in an increase of logarithm of odds from 2.2 to 5.3. This increase was estimated to correspond to a $P$ value <0.0016 using permutation. The transcription factor PAX4 is located at 7q32 and participates downstream of ISL1 in the transcription factor cascade critical to β-cell development. Association with type 1 diabetes was also observed using the transmission disequilibrium test for two haplotypes at the PAX4 locus ($P < 0.05$). We conclude that pathway-restricted linkage analysis assists in the identification of possible gene-gene interactions and that 5q11-q13 and 7q32 together constitute a significant susceptibility factor for type 1 diabetes. *Diabetes* 53:1584–1591, 2004**

Type 1 diabetes is a common multifactorial disease characterized by autoimmune destruction of the insulin-producing β-cells in the endocrine pancreas, resulting in deranged metabolic homeostasis with raised blood glucose concentration. A simple pattern of inheritance has not been identified for type 1 diabetes. The susceptibility to type 1 diabetes is partially genetically determined, as indicated by an increased familial aggregation, with a relative risk of 15 for siblings ($\lambda_s$) to patients, and a higher concordance rate among monozygotic than dizygotic twins (1). The major susceptibility locus for type 1 diabetes is the major histocompatibility complex on chromosome 6q21.3. The insulin gene on chromosome 11p15 and the CTLA4 gene on chromosome 2q33 (2,3) are also established susceptibility genes. In addition, several other chromosome regions have been identified through genome-wide linkage analysis (4,5).

In a genome-wide linkage analysis for type 1 diabetes in families from Scandinavia, chromosome region 5p13-q13 provided evidence for suggestive linkage (logarithm of odds [LOD] 2.2, $P < 0.0008$, $\lambda_s = 1.25$) (5). The insulin-enhancer binding protein 1 (ISL1) gene is an obvious candidate gene in this region. ISL1 is a transcription factor with an important role during the development of the pancreas (6). ISL1 is expressed in endocrine and exocrine cells and the central nervous system. Furthermore, it binds to the promoter/enhancer regions and affects the transcription of, e.g., the insulin, glucagon, somatostatin, and amylin genes (7). In mouse embryos lacking ISL1, development of the pancreas is arrested at embryonic day 9.5 (E9.5) (8); the endocrine cells are missing with a complete loss of differentiated islet cells (9).

In this report, we describe an investigation of association and gene-gene interactions involving ISL1 in relation to type 1 diabetes. Since the genetic risk for type 1 diabetes is likely due to interaction between several susceptibility genes in the same biochemical pathway, we have performed a pathway-restricted linkage analysis to detect such interactions. We study the interactions between the ISL1 locus and chromosome regions harboring other transcription factor genes involved in the development and maintenance of the endocrine pancreas.

From the [1]Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; and the [2]Department of Endocrinology, Lund University, Malmö, Sweden.

Address correspondence and reprint requests to Dr. Ingrid Kockum, Department of Molecular Medicine, Karolinska Institutet, CMM L8:00, Karolinska Hospital, S-171 76 Stockholm, Sweden. E-mail: ingrid.kockum@cmm.ki.se.

Received for publication 21 May 2003 and accepted in revised form 15 March 2004.

A list of steering committee members for the European Consortium for IDDM Genome Studies appears in the APPENDIX.

ETDT, extended transmission disequilibrium test; LD, linkage disequilibrium; LOD, logarithm of odds; MS, multiple sclerosis; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test; UTR, untranslated region; WRS, Wolcott-Rallison syndrome.

TABLE 1
Transcription factors and other key genes involved in the development and maintenance of the endocrine pancreas

| Gene | Chromosome | Closest marker | Estimated distance between marker and gene | Multipoint LOD at D5S407 | |
|---|---|---|---|---|---|
| | | | | Positive | Negative |
| PBX1 | 1q23 | D1S196 | 3 cM | 1.13 | 1.25 |
| NeuroD/β2 | 2q32 | D2S152 | 2.5 cM | 0.00 | 2.38 |
| NKX6.1 | 4q21.2-22 | D4S1538 | 0.8 cM | 2.07 | 0.24 |
| ISL1 | 5p11-q13 | D5S407 | 5 cM | ND | ND |
| PAX4 | 7q32 | D7S530 | 1.7 cM | 5.26 | 0.20 |
| NGN3 | 10q21.3 | D10S537 | 2.0 cM | 0.42 | 1.51 |
| INS | 11p15.5 | TH | 8,000 bp | 2.00 | 0.58 |
| | | D11S904 | 4 cM | 0.89 | 0.61 |
| PAX6 | 11p13 | D11S907 | 1 cM | 0.38 | 2.04 |
| | | D12S366 | 1 cM | 0.40 | 1.11 |
| HNF1A | 12q24.2 | D12S342 | 4 cM | 0.00 | 2.02 |
| IPF1/PDX1 | 13q12.1 | D13S192 | 0.2 cM | 0.00 | 2.74 |
| HNF6A | 15q21.1-q21.2 | CYP19 | 1 cM | 2.06 | 0.53 |
| | | D15S114 | 0.1 cM | 0.75 | 1.02 |
| ISL2 | 15q23 | D15S125 | 1 cM | 0.99* | 1.25 |
| HNF3B | 20p11 | D20S112 | 5 cM | 0.32 | 1.75 |
| IA-1 | 20p11 | D20S112 | 3 cM | 0.32 | 1.75 |
| | | D20S107 | 4 cM | 0.00 | 3.13 |
| HNF4A | 20q12-q13.1 | D20S178 | 3 cM | 0.46 | 1.36 |

Genotypes used for this interaction analysis were produced during a genome-wide scan for type 1 diabetes susceptibility genes (5). The interaction analysis was not presented in this report. *LOD for positive multipoint interaction between D15S125 and chromosome 5 was 3.2 at D5S647. ND, not determined.

## RESEARCH DESIGN AND METHODS

The pathway-restricted linkage analysis was performed in 408 multiplex families from Scandinavia that were used in a previously published genome-wide linkage study (5). For the ISL1 gene sequencing and the ISL1 and PAX4 single nucleotide polymorphism (SNP) analyses reported here, 196 Swedish families (186 muliplex and 10 simplex) were used. Twelve unrelated patients, selected from the families with the strongest evidence for linkage to chromosome 5p13-q13, and 4 healthy unrelated control subjects were used for sequence analysis of ISL1. All six exons in the ISL1 gene were sequenced (exon 1, 264 bases; exon 2, 190 bases; exon 3, 260 bases; exon 4, 287 bases; exon 5, 168 bases; and exon 6, 1,226 bases [out of which 1,111 bases are 3′ untranslated region {UTR}]). In addition, 226 bases of the promoter region and a total of 1,889 bases of introns surrounding the exons were sequenced. DNA sequencing was performed with BigDye Terminator version 2.0 (Applied Biosystems) and analyzed in Sequencing Analysis Software version 3.3 (Applied Biosystems) and SeqScape version 1.0 (Applied Biosystems).

SNPs were analyzed by PCR amplification and sequencing (Pyrosequence, Uppsala, Sweden). For each SNP, seven DNA samples were used for verification and quality control. The SNPs were determined following standard protocol from the vendor and analyzed in version 1.1 or 1.2 AQ of the PSQ96 SNP Software. Quality control involved checking that the amplified DNA could not prime sequence itself by DNA looping, primer-dimers could not prevent the sequencing process, and the predicted sequences were actually identified. Microsatellites were analyzed using standard methods as described previously (5). SNPs in the PAX4 gene that passed the quality controls and those with a rare allele frequency between 0.05 and 0.5 in 94 unrelated Swedish individuals were accepted for further study. Mendelian consistency, excess of homozygosity, and allele frequency were analyzed with the zGenStat program (H. Zazzi, unpublished). Using the Arlequin program (10), we showed that each ISL1 and PAX4 marker individually were in Hardy-Weinberg equilibrium; however, the haplotype frequencies were not ($P < 0.0001$), with fewer heterozygotes than expected. The TRANSMIT program was used for transmission disequilibrium test (TDT) analysis (11). Conditional extended TDT (ETDT) analyses were performed using the UNPHASED program (12). *P* values were not corrected for multiple comparisons.

Interactions between chromosome 5 and markers at other loci were performed with ALLEGRO (13). We used family-specific LODs from 17 markers mapping close to transcription factor genes involved in the development and maintenance of the endocrine pancreas (Table 1). Interaction analyses with *1*) positive (epistasis) and *2*) negative (heterogeneity) weighting were performed. Thus, for the positive weighting, the LOD for each family at a selected marker on another chromosome was used as a positive weight when calculating LODs on chromosome 5. Families with LOD ≤0 were given a weight of 0.

To estimate the significance of the results of the interaction analysis, we performed 10,000 permutations of positive weights, randomly selected from the markers used in the genome scan (5). In each permutation, 17 markers (Table 1) were randomly selected from 291 markers that remained after excluding those from chromosome X and 50 cM on either side of D5S407. In each permutation, the weights were randomly assigned to families. The resulting 17,000 weight files were then used to perform linkage analysis of a segment of chromosome 5 (50 cM to either side of D5S407). We then counted how many permutations of the 17 weights that contained at least one linkage peak with a LOD >5.26.

Association between markers was estimated by constructing haplotypes in SIMWALK2 (14) and estimating D′ using the haploxt command in GOLD (15).

## RESULTS

In the genome scan for type 1 diabetes susceptibility genes, we found suggestive linkage (LOD 2.2) to chromosome region 5p13-q13 (5). To validate the public genome sequence in the 5p13-q13 region, we assembled a "virtual physical map" by connecting bacterial artificial chromosome clones and checked that known genetic markers map to the predicted region. The resulting order corresponds well with the utilized assembly of the National Center for Biotechnology Information human genome (build 31).

This analysis confirmed that the markers linked to type 1 diabetes on chromosome 5 were located in the 5q11-q13 region. In the published genome scan (5), the highest multipoint was observed at marker D5S407, located 9.6 Mb from the centromeric end of p-arm of chromosome 5. ISL1 is located on the q-arm of chromosome 5, 168 kb from the D5S822 (Fig. 1). Since the transcription factor ISL1 is involved in the development of the pancreas, has a binding site in the insulin promoter, and is located close to the linkage peak, it was considered a candidate gene for type 1 diabetes susceptibility. All six exons of the gene, the

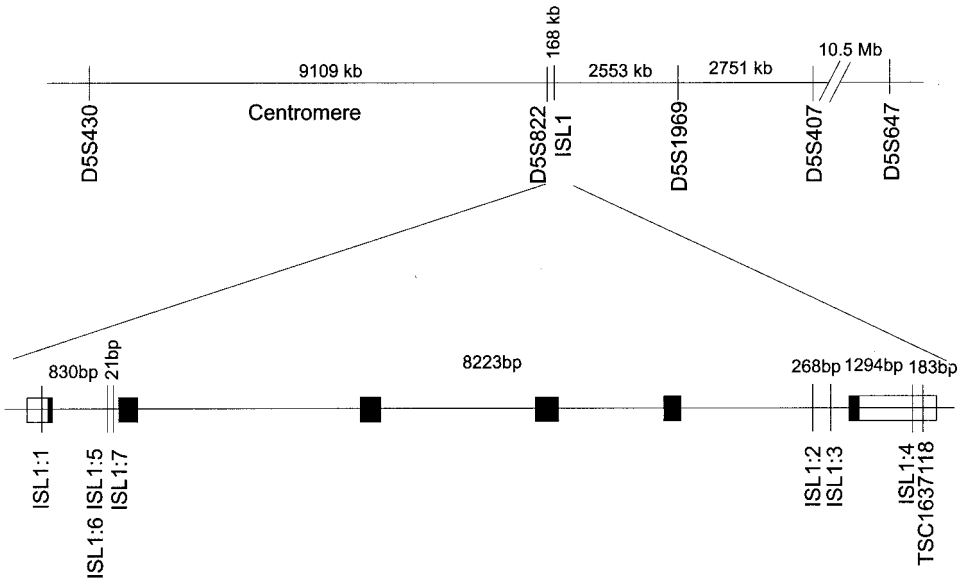


**FIG. 1. Location of ISL1 gene and markers on chromosome 5p11-q13 used in this investigation. The distances in bases are shown between markers. TSC1637118 was identified by sequencing but not analyzed in our families. In the lower part of the figure, the 5′- and 3′UTRs of the ISL1 gene are shown as white boxes and exons as black boxes. ISL1:1 = rs3917084, ISL1:2 = TSC0312199, ISL1:3 = TSC0312200, ISL1:4 = TSC0424949, ISL1:5 is a newly discovered microsatellite marker (see Table 2), ISL1:6 = rs4151674, and ISL1:7 = rs4151675.**

promoter region, intron 1, and intron 5 were sequenced in 12 type 1 diabetic patients and 4 individuals without type 1 diabetes. Eight polymorphisms were observed. Seven of these were selected for genotyping in the Swedish families. One SNP was located in the 5′UTR (ISL1:1, rs3917084), two SNPs in intron 5 (ISL1:2, TSC0312199 and ISL1:3, TSC0312200), and a fourth SNP was located in the 3′UTR (ISL1:4, TSC0424949) (Fig. 1). Two of the SNPs (ISL1:6, rs4151674 and ISL1:7, rs4151675) were found in intron 1 in a sequence rich in CAAA repeats. We also identified a microsatellite (ISL1:5) with three alleles (WT, WT-4bp, and WT+8bp) in this region (Table 2). The eighth SNP was found in the 3′UTR (TSC1637118). This is a poly-A repeat where an extra A was present in three patients and one control subject. It was excluded for technical reasons. The variations found in the ISL1 gene were all in noncoding sequences. No significant associations could be detected with TDT between type 1 diabetes and any of the individual SNPs (data not shown). However, with global TDT analysis for haplotypes based on the SNP markers (11), a $P$ value of 0.03 was observed for association with haplotypes composed of ISL1:2-ISL1:3, ISL1:1-ISL1:2-ISL1:3, and ISL1:1-ISL1:5-ISL1:6-ISL1:7-ISL1:2-ISL1:3 (Table 3). The global TDT analysis gives a summary $P$ value for all haplotypes composed of the selected markers. An attempt to identify the effects of individual haplotypes from the ISL1 is presented in Table 4. Three ISL1 haplotypes were negatively associated with type 1 diabetes: ISL1:1-ISL1:5-ISL1:6-ISL1:7-ISL1:2-ISL1:3-ISL1:4, T-WT-A-A-G-C-A ($P < 0.02$), and T-WT-A-A-G-C-T and T-WT-G-A-G-C-T (both $P < 0.04$), whereas one haplotype, C-WT-A-A-G-T-T, was positively associated. However, for the individual haplotypes, the expected number of transmissions was small (<5 for the negatively and 28 for the positively associated haplotypes).

Linkage disequilibrium (LD), estimated by D′, was observed among all ISL1 SNP markers (D′ >0.61, $P < 1 \times 10^{-4}$) (Table 5) except ISL1:1-ISL1:5, ISL1:1-ISL1:6, ISL1:5-ISL1:6, ISL1:5-ISL1:2, ISL1:5-ISL1:3, ISL1:5-ISL1:4, ISL1:6-ISL1:2, ISL1:7-ISL1:2, ISL1:7-ISL1:3, and ISL1:7-ISL1:4. Significant evidence of LD was observed for intermarker distances up to 9,657 kb (D′>0.39, $P < 1 \times 10^{-4}$) (Table 5). In addition, evidence of LD across the centromere was observed for D5S822 paired with any of the ISL1 markers except ISL1:7 (D′ 0.39–0.97, $P < 0.04$) and for D5S430 and ISL1:1 (D′ 0.32, $P < 0.004$) (Table 5).

Given the possible association between ISL1 and type 1 diabetes, we performed a pathway-restricted interaction analysis to study interactions between genetic locations harboring transcription factors important to pancreas development and maintenance. Table 1 shows a list of genes that are involved in the ISL1 pathway and/or are implicated in the development or maintenance of the endocrine pancreas. The closest microsatellite marker to each of the genes was selected for analysis of interaction with ISL1. The chromosome region 5q11-q13 interacted with marker D7S530 located at 7q32. Linkage analysis in the Scandinavian families, allowing for interaction between 7q32 and 5q11-q13, resulted in an increased multipoint LOD from 2.2 to 5.3 for 5q11-q13, using positive weighting based on the family-specific LODs for linkage at 7q32 (Fig. 2).

We attempted to estimate the significance of this observed increase in LOD by permutation. We randomized the weights from 17 markers selected from 291 markers used in the genome scan with respect to the family number. This was followed by linkage calculation at 5q11-q13 using these weights when summing the linkage evidence over families. This process was repeated 10,000 times. The highest observed LOD in this analysis was 6.54. In 16 permutations, one LOD above 5.3 was observed. This gives a $P$ value of 0.0016 for our interaction.

The gene for transcription factor PAX4 is located 1.2 Mb (1.7 cM) from the D7S530 marker at 7q32, and the promoter region of PAX4 has a binding site for ISL1 4,466 bases 5′ of the translation initiation codon (16). Seven SNPs were initially selected in the PAX4 gene. Three of

these SNPs passed the quality controls and were heterozygous in our families (PAX4-1: TSC0207823; PAX4-2: TSC0119990; and PAX4-3: TSC0090903) and were genotyped in the Swedish families. We observed a LOD of 0.4 for positive weighting, and 0.5 for negative weight at 5q11-q13, using multipoint LOD in the PAX4 gene. But when analyzing for interaction between 5q11-q13 and the D7S530 microsatellite marker, the LOD for the positive weight is only 0.6 in the Swedish families. None of the PAX4 SNPs show association with type 1 diabetes individually. However, PAX4 showed association ($P < 0.02$) with type 1 diabetes, using global TDT analysis for haplotypes consisting of all three SNPs (Table 3). The T-C-C haplotype is negatively associated ($P < 0.001$), whereas the C-T-C haplotype is positively associated, with type 1 diabetes ($P < 0.05$) (Table 4). In addition, the haplotypes PAX4:1-PAX4:2 (T-C) and PAX4:2-PAX4:3 (C-C) are associated with type 1 diabetes ($P < 0.003$ and $P < 0.01$, respectively, data not shown). The PAX4 C-T-C haplotype is present among patients in 156 Swedish families. In these families, the LOD on chromosome 5 reaches 2.8, while it is only 0.19 in the families without the PAX4 C-T-C haplotype.

Clear LD between the SNPs within PAX4 was observed ($D' > 0.82$, $P < 1 \times 10^{-5}$) (Table 5), and weak association was observed between the PAX4:1 SNP and microsatellite markers D7S530 ($D'$ 0.16, $P < 0.04$) and CFTR ($D'$ 0.12, $P < 0.05$).

A conditional ETDT analysis looking at association of the PAX4:1-PAX4:2-PAX4:3 marker combination while conditioning on the ISL1:1-ISL1:5-ISL1:6-ISL1:7-ISL1:2-ISL1:3-ISL1:4, T-WT-A-A-G-C-A, T-WT-A-A-G-C-T, T-WT-G--G-C-T, or C-WT-A-A-G-T-T haplotypes revealed no significant associations. The same tests conditioning on the PAX4:1-PAX4:2-PAX4:3, C-T-C, and T-C-C haplotypes showed no significant association to the ISL1:1-ISL1:2-ISL1:3-ISL1:4 marker combination.

The second highest observed LOD in the pathway-restricted interaction analysis was obtained using positive weights from D15S125, resulting in an LOD of 3.2 on chromosome 5 at the marker D5S647. This marker is placed 10 cM (10.5 Mb) from D5S407 and within the 1-LOD support interval for the chromosome 5 locus in the initial genome-wide linkage study (5). The $P$ value for this observation was estimated to 0.13 by simulation (see above). ISL2 maps only 10 Mb away from the D15S125 marker. Similarities in sequence and expression patterns between ISL1 and ISL2 indicate that they have similar functions (17,18).

TABLE 2
Haplotypes in intron 1 of the ISL1 gene

| Haplotype | ISL1:5 | ISL1:6 | ISL1:7 | Observed (non–type 1 diabetes/ type 1 diabetes) | Sequence, starting at intron position + 695, counting first base after first exon as +1 |
|---|---|---|---|---|---|
| 1 | WT | A | A | 565/561 | GCAATGCTCTAAAAAAAAAAAAAAGAAAGAAAGAAAGAAAAAAACCTCCCAGAGTA* |
| 2 | WT | G | A | 36/25 | ----------G----------* |
| 3 | WT-4bp | A | A | 10/8 | ----------* |
| 4 | WT+8bp | G | G | 1/2 | ----------GAAAGAAA----------G----------* |

*This position is 86 bp from the 5′ end of exon 2. No significant difference in frequency of the different haplotypes between patients and control subjects was observed.

## DISCUSSION

We have previously reported suggestive linkage between type 1 diabetes and the chromosome 5p13-q13 region in Scandinavian type 1 diabetic families (LOD 2.2, $P < 8 \times 10^{-4}$, $\lambda_s = 1.25$) (5). ISL1 was placed within the 1-LOD support interval and selected as an obvious functional candidate for further analysis. Six SNPs and one microsatellite marker in the gene were genotyped in 196 Swedish type 1 diabetic families. This indicated weak association of ISL1 to type 1 diabetes. Given the magnitude of linkage, there are probably additional type 1 diabetes susceptibility genes in the 5q11-q13 region. We also found evidence for interaction between the 5q11-q13 and the

TABLE 3
Familial association between ISL1 and PAX4 haplotypes

| Gene | Markers included in haplotype | Global TDT* *P* value | Number of families with transmissions to affected offspring |
|---|---|---|---|
| ISL1 | ISL1:2-ISL1:3 | 0.03 | 209 |
| ISL1 | ISL1:1-ISL1:2-ISL1:3 | 0.04 | 208 |
| ISL1 | ISL1:1-ISL1:5-ISL1:6-ISL1:7-ISL1:2-ISL1:3 | 0.04 | 193 |
| PAX4 | PAX4:1-PAX4:2 | 0.02 | 209 |
| PAX4 | PAX4:2-PAX4:3 | 0.05 | 205 |
| PAX4 | PAX4:1-PAX4:2-PAX4:3 | 0.02 | 203 |

*TDT analysis was performed using the TRANSMIT program.

7q32 regions with an approach coined pathway-restricted linkage analysis.

ISL1 is a good candidate for type 1 diabetes, since it is involved in both the development and maintenance of differentiated functions of insulin-producing β-cells. Type 1 diabetes is caused by autoimmune destruction of β-cells. In a healthy individual, there is a presumed balance, within the framework of the normal age-dependent deterioration of β-cell function, of genes causing destruction of the β-cell and those responsible for regeneration and robustness. Thus, any gene product that deranges the function and development of the pancreatic β-cells or the expression of insulin would be a candidate susceptibility gene for type 1 diabetes. Consequently, diabetes could result from either increased autoimmune activity or loss of the capacity to regenerate β-cells and thus diminished robustness to attack or from a combination of these opposing processes. To date, the identified susceptibility genes for type 1 diabetes are thought to be involved in autoimmune reactions (e.g., HLA, insulin, and CTLA4). This does not exclude that some type 1 diabetes susceptibility genes affect robustness and/or development of β-cells. In fact, insulin could theoretically affect both the robustness and/or development of the β-cell and be involved as an autoantigen in type 1 diabetes. Mutations in the eukaryotic translation initiation factor 2-a kinase 3 (EIF2AK3) gene, which is highly expressed in the pancreatic islet and affects protein synthesis, results in Wolcott-Rallison syndrome (WRS) (19). WRS does not have an autoimmune etiology, but the permanent dependency on insulin suggests that biological processes involved in WRS may be relevant to type 1 diabetes (19). Hence, genes involved in the maintenance of the integrity of the pancreatic β-cell are also candidates for type 1 diabetes susceptibility.

The transcription factor ISL1 belongs to the LIM protein family, which contains a COOH-terminal DNA binding homeodomain and two tandemly repeated Cys-His motifs termed LIM domains (20). ISL1 is expressed in, and required for, the development of endocrine islet cells, as well as for the regulation of hormone expression in these cells. ISL1 is also involved in exocrine cell differentiation in the dorsal bud (8,9). Pancreatic endocrine cells start to express ISL1 before the expression of hormones, which suggests that ISL1 regulates the expression of endocrine hormones (9). In the insulin promoter, ISL1 has been shown to bind the A3 and A1 enhancer elements (20).

ISL1 is also expressed in the nervous system (21), where it is required for the generation of motor neurons (8). Multiple sclerosis (MS) has been reported to be linked to 5q11 ($P < 0.009$) (22). Due to this colocalization of susceptibility loci, it is possible that the same gene is responsible for chromosome 5–linked susceptibility in both MS and type 1 diabetes. Since *ISL1* is expressed in the target tissues for both MS and type 1 diabetes and is important for the development and maintenance of these tissues, it is a possible common susceptibility gene for both type 1 diabetes and MS.

The chromosome region 5q11-q13 has not been linked to type 1 diabetes in any patient material other than the Scandinavian families. However, the power has not been large enough in other investigations to exclude linkage to this region for a locus with the observed locus-specific risk

TABLE 4
Association analysis of ISL1 and PAX4 SNP haplotypes

| Gene | ISL1:1 | ISL1:5 | ISL1:6 | ISL1:7 | ISL1:2 | ISL1:3 | ISL1:4 | TDT* *P* value | Transmission (%) | Observed number of transmissions | Expected number of transmissions |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ISL1 | T | WT | A | A | G | C | A | 0.02 | 16.1 | 0.66 | 2.08 |
| ISL1 | T | WT | A | A | G | C | T | 0.04 | 30.6 | 1.04 | 1.70 |
| ISL1 | T | WT | G | A | G | C | T | 0.04 | 0.16 | 0 | 1.26 |
| ISL1 | C | WT | A | A | G | T | T | 0.02 | 65.0 | 36.9 | 28.4 |
| Gene | PAX4:1 | PAX4:2 | PAX4:3 | | | | | TDT* *P* value | | | |
| PAX4 | T | C | C | | | | | 0.001 | 6.6 | 0.59 | 4.5 |
| PAX4 | C | T | C | | | | | 0.05 | 52.9 | 330.9 | 312.5 |

*TDT analysis was performed using the TRANSMIT program. Because the number of transmissions are also estimated when there is not complete information in the family, the number of observed transmissions are not always integers. For ISL1 markers, haplotypes formed by 2, 3, 4, 5, and 6 markers were also analyzed, which resulted in the same haplotypes being associated as shown above. For PAX4 markers, haplotypes formed by two markers were also analyzed, resulting in the same haplotypes being associated as shown above.

TABLE 5
LD estimates between markers close to ISL1 and PAX4 in 196 Swedish type 1 diabetic families

| Marker 1 | Marker 2 | Distance between markers (kb) | No. | df | $\chi^2$ | *P* value (<) | D′ |
|---|---|---|---|---|---|---|---|
| Chromosome 5p11-q13 | | | | | | | |
| D5S430 | ISL1:1 | 9,657 | 600 | 3 | 13.3 | 0.004 | 0.32 |
| D5S822 | ISL1:1 | 168 | 601 | 4 | 347.6 | 0.0001 | 0.97 |
| D5S822 | ISL1:2 | 177 | 605 | 4 | 97.2 | 0.0001 | 0.39 |
| D5S822 | ISL1:3 | 178 | 608 | 4 | 116.4 | 0.0001 | 0.43 |
| D5S822 | ISL1:4 | 179 | 382 | 4 | 62.4 | 0.0001 | 0.40 |
| D5S822 | ISL1:5 | 169 | 567 | 4 | 10.2 | 0.04 | 0.84 |
| D5S822 | ISL1:6 | 169 | 545 | 4 | 110.8 | 0.0001 | 0.79 |
| ISL1:1 | ISL1:2 | 9.1 | 593 | 1 | 45.8 | 0.0001 | 1.00 |
| ISL1:1 | ISL1:3 | 9.3 | 596 | 1 | 40.7 | 0.0001 | 0.95 |
| ISL1:1 | ISL1:4 | 11 | 376 | 1 | 19.5 | 0.0001 | 0.83 |
| ISL1:5 | ISL1:7 | 0.02 | 545 | 1 | 97.5 | 0.0001 | 1.00 |
| ISL1:6 | ISL1:7 | 0.02 | 548 | 1 | 33.5 | 0.0001 | 1.00 |
| ISL1:6 | ISL1:2 | 8.2 | 538 | 1 | 16.2 | 0.0001 | 0.61 |
| ISL1:6 | ISL1:3 | 8.5 | 535 | 1 | 18.6 | 0.0001 | 0.65 |
| ISL1:6 | ISL1:4 | 9.8 | 426 | 1 | 23.8 | 0.0001 | 0.85 |
| ISL1:2 | ISL1:3 | 0.3 | 600 | 1 | 476.4 | 0.0001 | 0.90 |
| ISL1:2 | ISL1:4 | 1.6 | 377 | 1 | 299.8 | 0.0001 | 0.89 |
| ISL1:3 | ISL1:4 | 1.3 | 377 | 1 | 325.8 | 0.0001 | 0.94 |
| Chromosome 7q32 | | | | | | | |
| CFTR | PAX4:1 | 10,128 | 609 | 3 | 8.1 | 0.05 | 0.12 |
| PAX4:1 | PAX4:2 | 1 | 557 | 1 | 371.1 | 0.0001 | 0.82 |
| PAX4:1 | PAX4:3 | 4 | 639 | 1 | 80.1 | 0.0001 | 0.94 |
| PAX4:1 | D7S530 | 2,851 | 613 | 4 | 10.2 | 0.04 | 0.16 |
| PAX4:2 | PAX4:3 | 3 | 611 | 1 | 70.5 | 0.0001 | 0.89 |

Only combinations of markers that show significant evidence of LD ($P < 0.05$) are shown.

($\lambda_s = 1.25$). Identification of association to ISL1 markers will now make it possible to use case-control materials to confirm the involvement of ISL1 in type 1 diabetes susceptibility. Although ISL1 has not been previously associated with type 1 diabetes, it has been investigated in type 2 diabetes and obesity. In a Japanese family with type 2 diabetes, a Q310X nonsense mutation in exon 5 of the ISL1 gene was reported (23). This variation could not be detected in the patients or control subjects who were sequenced in our studies. However, no linkage could be found to ISL1 in French, African-American, or Nigerian type 2 diabetic families (24,25), and ISL1 could not be linked with obesity (26). In morbidly obese subjects, carriers of the rs3917084 (ISL1:1) G allele in the promoter region showed decreased risk for type 2 diabetes (26). This allele has a frequency of 0.05 in the Swedish families and is not in itself significantly associated with type 1 diabetes.

Based on the presented data, we believe that ISL1 is either in LD with another susceptibility gene or that it is only one of several susceptibility genes in this region of linkage. There are many examples of susceptibility loci for multifactorial diseases that have turned out to be due to several susceptibility genes mapping close to each other: Niddm1 in the GK rat consists of two susceptibility loci, Niddm1b and Niddm1i (27), and in the NOD mouse, Idd3, Idd5, Idd9, and Idd10 have all been shown to contain at least two susceptibility loci (28–31). Of note in this context is the presence of several type 1 diabetes susceptibility genes in the major histocompatibility complex region (32).

Type 1 diabetes is not only multigenetic but also probably due to gene-gene interaction between susceptibility genes. We therefore attempted a pathway-restricted interaction analysis to test the hypothesis that chromosome regions containing key genes for the function and maintenance of the pancreas may interact with ISL1 at 5q11-q13.

In our interaction analysis, using markers mapping close to transcription factor genes involved in the development and maintenance of the endocrine pancreas, we demonstrated interaction between 5q11-q13 and chromosome 7q32. This interaction analysis was carried out in a similar fashion to that done for the NIDDM1 locus and the CYP19 region in type 2 diabetes (33). The weighting we used was similar to their $\text{weight}_{\text{PROP}}$ (positive weight in our calculations) and $\text{weight}_{(1\text{-}0)}$ (negative weight in our calculations), which model positive interactions (such as epistasis) and heterogeneity, respectively. We estimated the significance of the observed increase in LOD for 5q11-q13 using permutation. The resulting *P* value is 0.0016 for the observed positive interaction between 5q11-q13 and 7q32. An alternative way to assess the significance is to employ a conservative $\chi^2$ test, since the increase in LOD multiplied by 2log(10) is asymptotically distributed as $\chi^2$ with 1 df (33). Using this test, the *P* value for the 5q11-q13 and 7q32 interaction was $<2 \times 10^{-4}$ after correcting for multiple comparisons ($n = 17$, $P < 0.004$).

The TDT analysis was carried out using the TRANSMIT computer program (11). This method has been developed to include information from more than one affected individual per family, which was important in our study because most of the families were multiplex. This TDT analysis can also deal with phase uncertainty for multilocus haplotypes. For this analysis, the resulting *P* value has

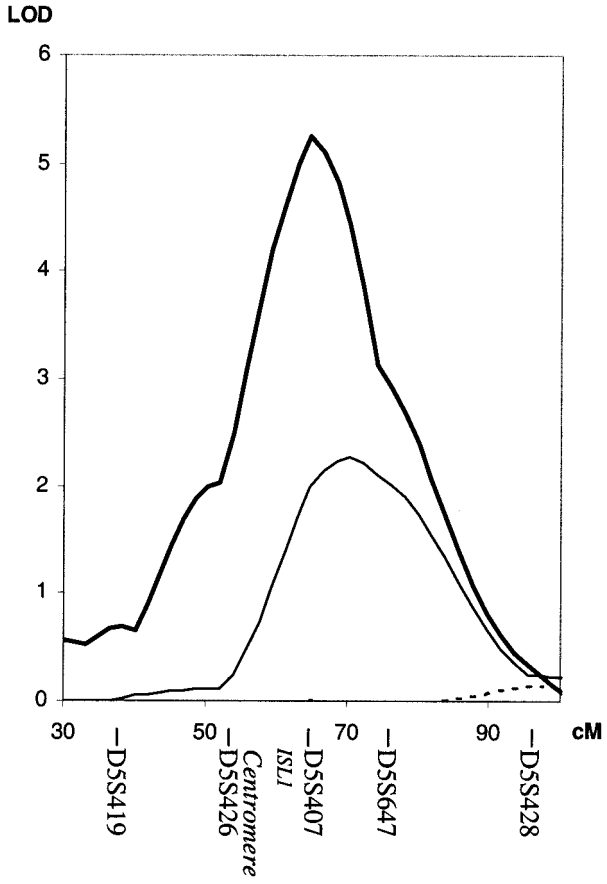


**FIG. 2. Linkage of type 1 diabetes to 50 cM on either side of D5S407 in the centromeric region of chromosome 5. The linkage contribution from each family was weighted by its LOD at D7S530 to generate a measure for interaction. The thin solid line denotes equal weight, thick solid line positive weight, and thin dotted line negative weight (see RESEARCH DESIGN AND METHODS for details). The markers used in the genome scan are indicated below the abscissa, and the centromere and the ISL1 gene are shown for location information.**

not been corrected for multiple comparisons; hence, the reported modest association requires confirmation.

We were not able to confirm the interaction between ISL1 and PAX4 by conditional ETDT analysis. This was, however, not surprising since we were unable to detect association to either the ISL1:1-ISL1:2-ISL1:3-ISL1:4 or PAX4:1-PAX4:2-PAX4:3 haplotypes using ETDT. This is presumably because larger families are broken up into trio families, thus leading to reduced power in the ETDT analysis compared with the TRANSMIT analysis.

The PAX4 gene maps to 7q32 and encodes a transcription factor with a paired-box domain. PAX4 plays an important role in the development and differentiation of the islet β- and δ-cells (16). Mice lacking *pax4* do not develop these cells and die shortly after birth from diabetes (34). A binding site for ISL1 is present in the PAX4 gene (16), and it has also been shown that PAX4 has an inhibitory effect on insulin gene transcription by binding to its promoter (35). Loss of PAX4 function may lead to an increased risk for diabetes because a missense mutation (R121W) in the PAX4 gene has been observed in Japanese type 2 diabetic patients at a frequency of 2% compared with 0% among control subjects. A homozygous carrier was reported with an early onset of diabetes who slowly fell into an insulin-dependent state without any signs of an autoimmune-mediated process (36). The same mutation has also been shown to be more frequent in Japanese subjects with LADA (late autoimmune diabetes in adults) than in control subjects (37). It is thus plausible that ISL1 and PAX4 interact to cause type 1 diabetes susceptibility in conjunction with genes for autoimmunity directed toward β-cells, each leading to a slight increase in risk for diabetes. The 5q11-q13 region does not appear to interact with either the insulin or the HLA genes, as there was no difference in the identity-by-descent sharing in sibpairs that carried different HLA genotypes or INS genotypes. Also, none of these groups showed increased evidence of linkage compared with all families (5).

We observed association between PAX4 haplotypes and type 1 diabetes, but we did not observe significant linkage between type 1 diabetes and the PAX4 region. This could, however, be because one of the associated PAX4 haplotypes (C-T-C) is common in the Swedish population (40%), which would make linkage harder to detect, just as has been observed for the IDDM2 region.

Although each of the ISL1 and PAX4 SNPs are in Hardy-Weinberg equilibrium, this is not observed for the ISL1 or PAX4 haplotypes. This could be because we are studying families with type 1 diabetes in which we also observe associated haplotypes. Thus, it would be of interest to test if these haplotypes obey Hardy-Weinberg equilibrium in control families.

The second strongest evidence for interaction with 5q11-q13 was chromosome 15p23. Since ISL2 maps to 15p23, it is plausible that the suggested interaction observed between these regions is due to an interaction between ISL1 and ISL2. The *P* value for the increase in LOD using the $\chi^2$ test was 0.03; after correction for multiple comparisons, this is no longer significant. We thus have to both prove that this interaction is real given the borderline *P* value and that ISL1 and ISL2 are responsible for the interaction between chromosome 5q11-q13 and 15p23.

In summary, we have shown evidence for association of ISL1 to type 1 diabetes or another gene in its vicinity. In addition, a gene mapping at 5q11-q13 interacts with a gene on 7q32, with PAX4 and ISL1 as possible candidates identified on functional grounds.

## ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Research Foundation International (1-1998-168 and 2-2000-570), the Swedish Research Council, the Novo Nordisk Foundation, the Swedish Strategic Funds, Svenska Diabetesförbundet, Barndiabetes Fonden, Smedbergs Stiftelse, Sven Järring Fonden, Torsten och Ragnar Söderbergs Stiftelser, Stiftelsen för vetenskapligt arbete inom diabetologin, Magnus Bergvalls Stiftelse, Albert Påhlssons Stiftelse, and Borgström & Hedströms forskningsfond.

The authors warmly thank Kristin Hellman and Mårten Jansson for technical advice. The Norwegian Diabetes Association and the ISID group (diabetes nurses) are acknowledged for their help in the collection of Norwegian families, and the Diabetes Incidence Study in Sweden and Childhood Diabetes registry are acknowledged for their help in the collection of Swedish families.

## APPENDIX

### Steering committee members for the European Consortium for IDDM Genome Studies

Christos Bartsocas (University of Athens, Faculty of Nursing, Children's Hospital, Athens, Greece), Gisela Dahlquist (Department of Paediatrics, Umeå University, Umeå, Sweden), Alberto de Leiva (Hospital De La Santa Creu I Sat Pau, University of Autónoma, Barcelona, Spain), Cécile Julier (Institut Pasteur, Paris, France), Mark Lathrop (Centre National de Génotypage, Evry, France), Holger Luthman (Department of Endocrinology, Lund University, Malmö, Sweden), Flemming Pociot (Steno Diabetes Center, Gentofte, Denmark), Kjersti S Rønningen (Laboratory of Molecular Epidemiology, National Institute of Public Health, Oslo, Norway), and Jørn Nerup (Steno Diabetes Center, Gentofte, Denmark).

## REFERENCES


1. Field LL: Genetic linkage and association studies of type 1 diabetes: challenges and rewards. *Diabetologia* 45:21–35, 2002
2. Bell GI, Horita S, Karam JH: A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* 33:176–183, 1984
3. Nistico L, Buzzetti R, Pritchard L, Van der Auwera B, Giovannini C, Bosi E, Larrad M, Rios M, Chow C, Cockram C, Jacobs K, Mijovic C, Bain S, Barnett A, Vandewalle C, Schuit F, Gorus F, Tosi R, Pozzilli P, Todd J: The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes: Belgian Diabetes Registry. *Hum Mol Genet* 5:1075–1080, 1996
4. Cox NJ, Wapelhorst B, Morrison VA, Johnson L, Pinchuk L, Spielman RS, Todd JA, Concannon P: Seven regions of the genome show evidence of linkage to type 1 diabetes in a consensus analysis of 767 multiplex families. *Am J Hum Genet* 69:820–830, 2001
5. Nerup J, Pociot F, the European Consortium for IDDM Studies: A genomewide scan for type 1-diabetes susceptibility in Scandinavian families: identification of new loci with evidence of interactions. *Am J Hum Genet* 69:1301–1313, 2001
6. Edlund H: Transcribing pancreas. *Diabetes* 47:1817–1823, 1998
7. Wang M, Drucker DJ: Activation of Amylin gene transcription by the LIM domain heomeobox gene isl-1. *Mol Endocrinol* 10:243–251, 1996
8. Pfaff SL, Mendelsohn M, Stewart CL, Edlund T, Jessell TM: Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. *Cell* 84:309–320, 1996
9. Ahlgren U, Pfaff SL, Jessell TM, Edlund T, Edlund H: Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature* 385:257–260, 1997
10. Schneider S, Roessli D, Excoffier L: *Arlequin Version 2000: A Software for Population Genetics Data Analysis*. Geneva, Genetics and Biometry Laboratory, University of Geneva, 2000
11. Clayton D: A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am J Hum Genet* 65:1170–1177, 1999
12. Dudbridge F: Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 25:115–221, 2003
13. Gudbjartsson DF, Jonasson K, Frigge ML, Kong A: Allegro, a new computer program for multipoint linkage analysis (Letter). *Nat Genet* 25:12–13, 2000
14. Sobel E, Lange K: Descent graphs in pedigree analysis: application to haplotyping, location scores, and marker-sharing statistics. *Am J Hum Genet* 58:1323–1337, 1996
15. Abecasis GR, Cookson WO: GOLD–graphical overview of linkage disequilibrium. *Bioinformatics* 16:182–183, 2000
16. Brink C, Chowdhury K, Gruss P: *Pax4* regulatory elements mediate beta cell specific expression in the pancreas. *Mech Dev* 100:37–43, 2001
17. Gong Z, Hui C, Hew CL: Presence of *isl-1*-related LIM domain homeobox genes in teleost and their similar patterns of expression in brain and spinal cord. *J Biol Chem* 270:3335–3345, 1995
18. Varela-Echavarria A, Pfaff SL, Guthrie S: Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem. *Mol Cell Neurosci* 8:242–257, 1996
19. Delépine M, Nicolino M, Barrett T, Golamaully M, Lathrop GM, Julier C: EIF2AK3, encoding translation initiation factor 2-a kinase3, is mutated in patients with Wolcott-Rallison syndrome. *Nat Genet* 25:406–409, 2000
20. Karlsson O, Thor S, Norberg T, Ohlsson H, Edlund E: Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* 344:879–882, 1990
21. Tsuchida T, Ensini M, Morton SB, Baldassare M, Edlund T, Jessell TM, Pfaff SL: Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* 79:957–970, 1994
22. The Transatlantic Multiple Sclerosis Genetics Cooperative: A meta-analysis of genomics screens in multiple sclerosis. *Mult Scler* 7:3–11, 2001
23. Shimomura H, Sanke T, Hanabusa T, Tsunoda K, Furuta H, Nanjo K: Nonsense mutation of islet-1 gene (Q310X) found in a type 2 diabetic patient with a strong family history. *Diabetes* 49:1597–1600, 2000
24. Vionnet N, Hani E, Lesage S, Philippi A, Hager J, Varret M, Stoffel M, Tanizawa Y, Chiu K, Glaser B, Permutt M, Passa P, Demenais F, Froguel P: Genetics of NIDDM in France: studies with 19 candidate genes in affected sib pairs. *Diabetes* 46:1062–1068, 1997
25. Tanizawa Y, Riggs AC, Dagogo-Jack S, Vaxillaire M, Froguel P, Liu L, Donis-Keller H, Permutt MA: Isolation of the human LIM/homeodomain gene islet-1 and identification of a simple sequence repeat polymorphism. *Diabetes* 43:935–941, 1994
26. Barat-Houari M, Clément K, Vatin V, Dina C, Bonhomme G, Vasseur F, Guy-Grand B, Froguel P: Positional candidate gene analysis of Lim domain homeobox gene (Isl-1) on chromosome 5q11-q13 in a French morbidly obese population suggests indication for association with type 2 diabetes. *Diabetes* 51:1644–1648, 2000
27. Galli J, Fakhrai-Rad H, Kamel A, Marcus C, Norgren S, Luthman H: Pathophysiological and genetic characterization of the major diabetes locus in GK rats. *Diabetes* 48:2463–2470, 1999
28. Wicker LS, Todd JA, Prins J-B, Podolin PL, Renjilian RJ, Peterson LB: Resistance alleles in two non-MHC-linked insulin dependent diabetes loci on chromosome3: Idd3 and Idd10 protects NOD mice from diabetes. *J Exp Med* 180:1705–1713, 1994
29. Hill NJ, Lyons PA, Armitage N, Todd JA, Wicker LS, Peterson LB: NOD Idd5 locus controls insulitis and diabetes and overlaps the orthologous CTLA4/IDDM12 and NRAMP1 loci in humans. *Diabetes* 49:1744–1747, 2000
30. Lyons PA, Hancock WW, Denny P, Lord CJ, Hill NJ, Armitage N, Siegmund T, Todd JA, Phillips MS, Hess JF, Chen SL, Fischer PA, Peterson LB, Wicker LS: The NOD Idd9 genetic interval influences the pathogenicity of insulitis and contains molecular variants of Cd30, Tnfr2, and Cd137. *Immunity* 13:107–115, 2000
31. Lyons PA, Armitage N, Lord CJ, Phillips MS, Todd JA, Peterson LB, Wicker LS: Mapping by genetic interaction: high-resolution congenic mapping of the type 1 diabetes loci Idd10 and Idd18 in the NOD mouse. *Diabetes* 50:2633–2637, 2001
32. Undlien DE, Lie BA, Thorsby E: HLA complex genes in type 1 diabetes and other autoimmune diseases: which genes are involved? *Trends Genet* 17:93–100, 2001
33. Cox NJ, Frigge M, Nicolae DL, Concannon P, Hanis CL, Bell GI, Kong A: Loci on chromosomes 2 (NIDDM1) and 15 interact to increase susceptibility to diabetes in Mexican Americans. *Nat Genet* 21:213–215, 1999
34. Huang HP, Tsai MJ: Transcription factors involved in pancreatic islet development. *J Biomed Sci* 7:27–34, 2000
35. Campbell SC, Cragg H, Elrick LJ, Macfarlane WM, Shennan KI, Docherty K: Inhibitory effect of pax4 on the human insulin and islet amyloid polypeptide (IAPP) promoters. *FEBS Lett* 463:53–57, 1999
36. Shimajiri Y, Sanke T, Furuta H, Hanabusa T, Nakagawa T, Fujitani Y, Kajimoto Y, Takasu N, Nanjo K: A missense mutation of Pax4 gene (R121W) is associated with type 2 diabetes in Japanese. *Diabetes* 50:2864–2869, 2001
37. Kanatsuka A, Tokuyama Y, Nozaki O, Matsui K, Egashira T: β-Cell dysfunction in late-onset diabetic subjects carrying homozygous mutation in transcription factors NeuroD1 and Pax4. *Metabolism* 51:1161–1165, 2002