Characterization of the Annexin I Gene and Evaluation of Its Role in Type 2 Diabetes

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Type 2 diabetes is a multifactorial, polygenic disorder characterized by chronic hyperglycemia resulting from pancreatic β-cell dysfunction and insulin resistance. Several monogenic forms of diabetes have been described (1), but the identification of predisposing genetic factors to the common form of type 2 diabetes has been difficult due to the heterogeneous nature of the disease. To identify susceptibility genes predisposing to type 2 diabetes, we performed a genome wide scan in 58 Finnish families comprising 440 individuals (229 with type 2 diabetes) and identified suggestive linkage using nonparametric linkage (NPL) analysis (NPL score 3.9, \(P < 0.0002\)) to chromosome 9p13-q21 (C.M.L., M.M. Mahtani, E. Widén, M.I. McCarthy, M.J. Daly, A. Kirby, M.P. Reeve, L. Krugylak, M. Lehto, T. Kanninen, P.A., T. Tuomi, L.C.G., and E.S. Lander, unpublished observations). Furthermore, this region has also been implicated in other genome wide scans for type 2 diabetes and/or related traits (2,3) and contains a number of putative candidate genes for type 2 diabetes, including annexin 1 (ANXA1) (4,5).

Human ANXA1 (also called calpactin II, lipocortin 1, or p35) belongs to the annexin superfamily, which consists of at least 11 different, abundant, and ubiquitously expressed proteins (6). In vitro studies have suggested that ANXA1 may play an important role in the regulation of both insulin secretion (6–12), including stimulation of insulin secretion by the arachidonic acid–phospholipase A2 (cPLA2) pathway (13,14) or in the signal cascades initiated from the insulin receptor (7,8). Given these considerations, ANXA1 is a good candidate gene for impaired insulin secretion, insulin resistance, and type 2 diabetes.

The schematic human and rat ANXA1 gene structures have been described (5,15) (GenBank accession nos. U25414 and X05908), but to enable mutation screening, we characterized the ANXA1 gene by sequencing all exons and flanking intron segments as well as the 5′- and 3′-untranslated regions (UTRs) (Fig. 1A). These sequences have recently been independently confirmed (16).

Subsequently, the minimal promoter (180 bp), all exons including 25 bp of the flanking introns, and the 341bp 3′ UTR were screened in 41 individuals with type 2 diabetes from our first study. Five single nucleotide polymorphisms (SNPs) were detected (Fig. 1B), including a silent A→G transition (Leu109Leu) in exon 5 and four SNPs in the noncoding parts of the gene. Three A→G transitions were found: one in the untranslated exon 1 (A58G), a second in intron 7, located 28 bp from the 5′ end of exon 8 (intron variance sequence (IVS)8–28A/G), and a third in intron 11, located 31 bp from the 3′ end of exon 11 (IVS11+31A/G). In addition, we found a T→G transition located 11 bp from the 5′ end of exon 12 (IVS12–11T/G).

Two of the SNPs, A58G and Leu401Leu, have been recently reported (17). An additional SNP, located in the 3′ UTR (C1197T), was reported in two chromosomes (17) but could not be detected in our sequencing of eight chromosomes.

All five SNPs were tested for association in a sample of...
197 Scandinavian unrelated parent-offspring trios (Table 1) with type 2 diabetes or abnormal glucose tolerance using the transmission disequilibrium test (TDT) (18). To increase the number of trios and the power in our study, we also included offspring with impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) (19). We only used one trio per family to test for association; linkage to this region has already been suggested in the same population (Lindgren et al., unpublished observations). The TDT analysis did not reveal any significant association with type 2 diabetes (Table 2). The IVS12-11T/G variant was too rare to allow further evaluation. The A58G variant showed a 22:12 transmission distortion of the common A allele to the offspring ($P = 0.12$). We also analyzed this variant in a sample of 481 pairs of case and control subjects matched for age, sex, and geographical origin (Table 1). No difference in either allele or genotype frequency was detected between the two groups ($P = 0.30$ and $P = 0.84$, respectively) (Table 3). The (CA)$_{15-25}$ repeat was genotyped in the 440 subjects of the initial linkage study, but no improvement of the original NPL score was observed in this analysis (data not shown).

**FIG. 1. A: ANXA1 exon/intron boundaries.** Lowercase letters designate the intronic sequences; uppercase letters designate the exonic sequences. The intron length varied from >6.5 kb of intron 1 to 93 bp of intron 2. The translation start (ATG) and the polyadenylation site (AATAAA) are marked with bold text. **B: The schematic structure of human ANXA1.** The transcription start (22) is indicated with an arrow (+1). The white areas represent the UTRs and the black areas represent translated regions. The five identified SNPs are viewed above the corresponding location. The figure is not drawn to scale.

**TABLE 1**
Clinical characteristics of subjects included in the TDT and case-control study

<table>
<thead>
<tr>
<th></th>
<th>TDT trio subjects</th>
<th>Case-control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male/female (n)</strong></td>
<td>99/98</td>
<td>252/229</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>39 ± 8</td>
<td>61 ± 10</td>
</tr>
<tr>
<td><strong>Age at diagnosis (years)</strong></td>
<td>35 ± 13</td>
<td>54 ± 11</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>28 ± 5</td>
<td>28 ± 5</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>5.9 ± 2.0</td>
<td>7.5 ± 1.8</td>
</tr>
<tr>
<td><strong>Fasting plasma glucose (mmol/l)</strong></td>
<td>7.7 ± 3.2</td>
<td>9.6 ± 3.8</td>
</tr>
<tr>
<td><strong>Plasma glucose at 2 h OGTT (mmol/l)</strong></td>
<td>9.3 ± 3.3</td>
<td>15.3 ± 5.5</td>
</tr>
</tbody>
</table>

Data are means ± SD unless otherwise indicated. Plasma glucose and serum insulin levels were measured at baseline (fasting) and 2 h after an oral glucose tolerance test (OGTT). Severe IGT is defined as 2-h plasma glucose values ≥9.6 mmol/l.
possible that we may have missed some ANXA1 gene variants. However, genotyping of the identified SNPs in both family trios (TDT) and case-control populations did not provide consistent evidence for association between ANXA1 and type 2 diabetes. Although we most likely identified most of the common SNPs in the screened parts of ANXA1, we cannot exclude the presence of additional SNPs in nearby regions. We therefore conclude that it is unlikely that the ANXA1 gene harbors variants that increase the risk of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. To ascertain families for genetic studies in type 2 diabetes, we worked with government-established health care centers in Finland and Sweden. All subjects underwent extensive phenotyping as previously described (20) and gave their consent to the study, which was also approved by the local ethics committee. Glucose tolerance status was assessed in accordance with the 1998 guidelines of the World Health Organization (1). Patients with known maturity-onset diabetes of the young 1–5 mutations, age at diabetes onset <18 years, GAD antibodies >5 reference index units, and/or fasting C-peptide levels <0.2 nmol/l were excluded (19).

Determination of the exon/intron boundaries of the ANXA1 gene. DNA from two healthy unrelated subjects was used for the determination of the exon/intron boundaries of the ANXA1 gene and to confirm the published cDNA sequence. Human genomic DNA extracted from lymphocytes was amplified using polymerase chain reaction (PCR) as previously described (21) (GeneAmp PCR system 9600; Perkin Elmer, Foster City, CA). The exon/intron boundary between intron 1 and exon 2 was determined with a Human Genome Walker Kit (Clontech, La Jolla, CA). This was performed in two steps: after the first PCR with an adapted primer 1 and one gene-specific primer (ANXA1-I2R), the product was amplified in a second “nested” PCR using adapter primer 2 and a second gene specific primer (ANXA1-I1R). The PCR product was then sequenced as described below.

The sequencing reactions were performed in both directions using the chain termination method with the Thermo Sequenase II Dye Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The sequencing products were separated on polyacrylamide gels using an AB373A or AB377 automated sequencer (Perkin Elmer). All results were analyzed using Sequencher 3.0 (Gene Codes, Ann Arbor, MI) by two independent readers.

Mutation screening of the ANXA1 gene by SSCP. A total of 41 subjects with type 2 diabetes from 26 families who presented excess allele sharing at chromosome 9 in the initial linkage study were chosen for the mutation screening of ANXA1. In these subjects and in two non-diabetic control subjects without family history of diabetes, the minimal promoter (22), the exons, and flanking intronic sequences were screened for mutations using the SSCP technique (23). All gels were scored manually by two independent readers, and all samples with a band pattern deviating from the expected band pattern (given from the two control subjects) were sequenced to find the causative variant. In contrast to the published cDNA sequence, a G instead of T at nucleotide position 362 from the transcription start site (exon 5) was observed in all individuals sequenced. This nucleotide change does not change the amino acid sequence of the protein (Leu) (5). The TATA box and the CAAT box are the major regulatory elements needed for transcription of the gene (15,22); both are included in the screened promoter segment.

Genotyping of identified variants. As linkage to the ANXA1 region has been suggested earlier, we only performed the test for association in the present study and therefore only one offspring per family was included. A total of 197 trios comprising 591 individuals were studied (Table 1). Of them, 101 offspring had type 2 diabetes, 65 had IGT, and 31 had IFG with normal 2-h blood glucose (1). We also selected an independent case-control replication sample from the same geographical area, including 481 patients with age at onset of type 2 diabetes >30 years or with severe IGT, i.e., a 2-h plasma glucose value >6.6 mmol/l during an oral glucose tolerance test. The control sample was made up of 481 age- and sex-matched nondiabetic subjects without family history of diabetes from the same geographical area (Table 1). Genotyping of the ANXA1 variants was performed using single base extension (SBE) with fluorescence polarization with modified protocols to allow high genotyping throughout (19). Genotypes were assigned by clustering data as previously described (19), and assignments were reviewed by at least two readers.

PCR and SBE primer sequences and PCR conditions used for this project are available on request or as an online appendix at http://diabetes.diabetesjournals.org.

Genotyping of the intron 11 (CA), repeat of the ANXA1 gene. A total of 440 individuals from the original linkage study (Lindgren et al. unpublished observations) were genotyped using the identified (CA)15–25 repeat in intron 11 of the ANXA1 gene. Genomic DNA (20 ng), prepared from peripheral blood lymphocytes, was amplified in 10-µl PCR, using a concentration of 2 µmol/l of primers (DSANX11I1P1ATGTTGAGAGTGTTAGGA,DSANX11I1R1-GGG TAAGTTGTCATATTTCAG). The forward primer was end-labeled with fluorescent dye (DNA Technology, Aarhus, Denmark). Thirty cycles of PCR were performed (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s), and 0.7 µl of each reaction was loaded onto a denaturing 6% polyacrylamide gel and electrophoresed for up to 4 h in 1 × Tris-borate-EDTA. The gels were run using ABI 377 Sequencers (Perkin Elmer) for fluorescent detection; two independent scorers read all gels, and discrepancies were subjected to a checking process, including repeating PCR for the entire family in question. Data were also checked for Mendelian segregation using the PEDMANAGER software (M.P. Reeve and M.J. Daly, personal communication).

Statistical analysis. All phenotype data in Table 1 are expressed as means ± SD. Transmission distortion from heterozygous parents was calculated using the McNemar test (18). P values for differences in allelic frequencies between case and control subjects were determined using the χ² distribution with one degree of freedom. Haplotype combinations of the five variants were tested for association with abnormal glucose tolerance using the χ² distribution with
REFERENCES


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