ORIGINAL ARTICLE

Fine mapping of a region on chromosome 21q21.11–q22.3 showing linkage to type 1 diabetes

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Background: Results of a Scandinavian genome scan in type 1 diabetes mellitus (T1D) have recently been reported. Among the novel, not previously reported chromosomal regions showing linkage to T1D was a region on chromosome 21.
Objective: To fine map this region on chromosome 21.
Methods and results: The linked region was initially narrowed by linkage analysis typing microsatellite markers. Linkage was significantly increased, with a peak NPL score of 3.61 (p = 0.0002), suggesting the presence of one or several T1D linked genes in the region. The support interval for linkage of 6.3 Mb was then studied by linkage disequilibrium (LD) mapping with gene based single nucleotide polymorphisms (SNPs). Thirty two candidate genes were identified in this narrowed region, and LD mapping was carried out with SNPs in coding regions (cSNPs) of all these genes. However, none of the SNPs showed association to T1D in the complete material, whereas some evidence for association to T1D of variants of the *TTC3*, *OLIG2*, *KCNE1*, and *CBR1* genes was observed in conditioned analyses. The disease related LD was further assessed by a haplotype based association study, in which several haplotypes showed distorted transmission to diabetic offspring, substantiating a possible T1D association of the region.
Conclusions: Although a single gene variant responsible for the observed linkage could not be identified, there was evidence for several combinations of markers, and for association of markers in conditioned analyses, supporting the existence of T1D susceptibility genes in the region.

Type 1 diabetes mellitus (T1D) [MIM 222100] is caused by an immune mediated destruction of the insulin producing β cells in the pancreas. β Cell destruction is irreversible and despite intensive insulin therapy the condition is connected with development of late diabetic complications and increased mortality.[1] T1D is characterised as a complex genetic disease, with multiple genetic loci and environmental factors involved. The main part of the genetic risk for T1D is conferred by the HLA region, but several "minor" loci are likely to be involved (reviewed by Pociot and McDermott[2]).

We have recently reported results of a Scandinavian genome scan in T1D comprising 464 affected sibling pairs from Denmark, Sweden, and Norway.[3] Previously published T1D loci were evaluated and new regions showing linkage to T1D were identified. Among the new, not previously reported chromosomal regions showing linkage to T1D was a region on chromosome 21.[3] This region mapped to the distal part of chromosome 21 (21q21.3–qTel) and revealed some evidence for linkage to T1D, with a maximum LOD score of 1.23 (p = 0.009) and a non-parametric linkage (NPL) score of 1.97 (p = 0.02). Linkage was most evident in the Danish population, where a maximum LOD score of 2.33 (p = 0.009) was obtained, with an NPL score of 2.64 (p = 0.004). Furthermore, interaction analyses have recently given additional support to this region as being important in T1D. In a combined neural network–decision tree analysis of Danish and Swedish T1D genome scan data, this region on chromosome 21 was also identified.[4]

The linked region on chromosome 21 comprised approximately 20 Mb, which equals about 35 cM (www.ncbi.nlm.nih.gov/genemap). The complete sequence of chromosome 21 is known and 225 genes have been identified on this chromosome.[5] The existence of the complete sequence of this chromosome means that new strategies for identification of genes are applicable instead of classical positional cloning. Furthermore the position of genes, microsatellites, and sequence variations can be identified.

The distal part of chromosome 21 harbours a few established candidate genes for autoimmunity, including T1D. The *AIRE* gene (21q22.3) encodes a regulator of transcription, in which mutations have been shown to give rise to the disease APECED (autoimmune poly-endocrinopathy-candidiasis-ectodermal dystrophy).[6–8] APECED is a rare systemic autoimmune disorder of monogenic and autosomal recessive inheritance.[9] Another gene is *CBR1* (21q22.12). Our group has carried out proteome analysis of interleukin 1β (IL-1β) exposed, diabetes-prone, Bio Breeding (BB-DP) rat islets of Langerhans in vitro.[10] We showed that the protein carbonyl reductase 1 (CBR1), encoded by the *CBR1* gene, is down-regulated fivefold by IL-1β exposure. As IL-1β is believed to be an important cytokine involved in destruction of the β cell,[11] this finding suggests a role for *CBR1* in T1D pathogenesis. Cu/Zn superoxide dismutase 1 (*SOD1*) (21q22.1), which is a potent antioxidant, has also been implicated in genetic predisposition to alloxan induced diabetes in mice.[12 13]

The runt related transcription factor 1 (*RUNX1*) (21q22.3), also called *AML1*, is another candidate gene for autoimmunity, including T1D. Recently, reports in three other autoimmune diseases—systemic lupus erythematosus, psoriasis, and rheumatoid arthritis—have demonstrated disease associated mutations in RUNX1 binding sites of three different genes.[14–16] However, in rheumatoid arthritis an intron 6 single nucleotide polymorphism (SNP) in the

Abbreviations: APECED, autoimmune poly-endocrinopathy-candidiasis-ectodermal dystrophy; cSNP, single nucleotide polymorphism in coding region; IL-1, interleukin 1; LD, linkage disequilibrium; LOD, log of odds ratio; NPL, non-parametric linkage; Sib-TDT, sibling transmission disequilibrium test; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test; T1D, type 1 diabetes

RUNX1 gene itself also showed disease association in a case–control study.[14]

On the basis of this combined information, we therefore believe that the rationale for fine mapping of the linked region is solid.

METHODS

DNA

We used DNA from 253 Danish T1D families (1097 individuals), comprising 155 sibling pair families and 98 simplex families.[3 17] For the initial linkage mapping, the 155 sibling pair families were used, whereas the subsequent linkage disequilibrium (LD) mapping included all the families. All probands were aged below 30 years at onset, and diagnosis of T1D was according to WHO criteria. Median age at onset was nine years.

Genotyping and linkage analysis of microsatellites

The microsatellites chosen were all dinucleotide repeats, and preferably the ones with the highest heterozygosity covering the region of interest most evenly. The markers were chosen based on the Marshfield map. Typing of microsatellites was done by polymerase chain reaction (PCR) amplification of marker sequences, using fluorescently labelled primers, followed by analysis of the fragments on automated DNA sequencing equipment (ABI Prism 3100, Applied Biosystems, Foster City, California, USA). Collection and analysis of data involved the software programs GeneScan, GenoTyper, and GeneMapper, while the multipoint NPL analysis and calculation of information content level of markers was carried out using the program GeneHunter version 1.2.[18] Marker order and intermarker distances were based on the physical map of chromosome 21.

Genotyping and analysis of SNPs

We identified all SNPs in coding regions (cSNPs) of the identified candidate genes, listed in dbSNP (NCBI), build 116. PCR primers were selected from the available sequences surrounding the mutations, and PCR products of 300 to 500 nucleotides in length were amplified. Single base primer extension reactions with primers terminating one nucleotide before the mutations were designed for each cSNP and employed for screening. We used the SnapShot Multiplex kit (Applied Biosystems) and automated DNA sequencing equipment (ABI prism 3100, Applied Biosystems). All SNPs were screened in 96 patients with T1D, and only SNPs with a frequency of the rarest allele of more than 3% were used for further analysis. The limit of 3% was set because these SNPs are believed to be common enough to have potential significance. The SNPs selected for further analysis were genotyped in the complete material of 253 Danish T1D families (1097 individuals). Depending on which technique yielded the best results, typing of the complete material was carried out by a PCR based restriction fragment length polymorphism (RFLP) assay or mutagenically separated (MS)-PCR, or otherwise by the SnapShot (primer extension) reaction.

Testing for linkage in the presence of linkage disequilibrium

Multiplex and simplex families were tested for linkage in the presence of linkage disequilibrium by Sib-TDT (sibling transmission disequilibrium test).[19] Subgroup analyses of families showing linkage and not showing linkage, respectively, were also done by Sib-TDT. Division of families with and without evidence of linkage was undertaken by evaluating the multipoint NPL score for each family at the position showing the maximum NPL score in the complete material. Families with evidence of linkage were defined as those with NPL scores above 1.00 (maximum score, 1.41). Analysis of transmissions of haplotypes was by the transmission disequilibrium test (TDT), using the GeneHunter software.

Pairwise linkage disequilibrium measurements

Parental (founder) haplotypes for microsatellites and genotyped SNPs in the 6.3 Mb linked region were estimated by maximum likelihood estimates, using GeneHunter vs 1.2. HaploXT (http://archimedes.well.ox.ac.uk/pise/haploxt-simple.html) to calculate pairwise LD indices (D′ values). LD values above 0.5 were defined as the threshold for LD.

RESULTS

Linkage analysis

Thirty five microsatellite markers covering the 20 Mb region of interest were identified and typed in 155 sibling pair families. Seven of the 35 markers were included in the original genome scan.[3] Data from the 35 markers were used in a multipoint NPL analysis (fig 1). The multipoint NPL analysis showed a significantly increased *z* value, with a peak NPL score corresponding to marker *D21S1920* of 3.61 ($p = 0.0002$). A 6.3 Mb region was identified as the ''one LOD drop'' interval, showing NPL scores above 2.61 (table 1). The information content level of the region of interest was between 95% and 100% (GeneHunter, version 1.2), based on data from the 35 markers (not shown). Thus it was not possible to narrow the region further by linkage analysis in the current families.

Linkage disequilibrium mapping

We have chosen a strategy of analysing the 32 identified positional candidate genes in order to characterise the region further and search for one or more aetiological mutations responsible for the demonstrated linkage of this region to T1D. The identified genes were examined by typing known cSNPs, identified from the dbSNP, build 116 (SNP database at NCBI, National Center for Biotechnology Information), in order to identify aetiological mutations or SNPs in linkage disequilibrium with a close by aetiological T1D mutation. In the 32 genes, 74 coding SNPs were identified. All 74 SNPs have been screened in 96 unrelated diabetic patients and only SNPs with a minor allele frequency above 3% were further genotyped and analysed for T1D association in the complete material of 253 T1D families. For a substantial number of SNPs no carriers were detected when screening 96 T1D individuals, questioning the relevance of these SNPs in the Danish population.

None of the SNPs analysed showed significant linkage, in the presence of linkage disequilibrium, to T1D (table 2). For one SNP, rs2154538—in exon 19 of the tetratricopeptide repeat domain 3 gene (*TTC3*)—a distorted transmission of the alleles to unaffected offspring was observed: 108 transmissions *v* 81 non-transmissions ($p = 0.0495$; uncorrected p value). Additionally, for rs2835655 in exon 39 of *TTC3*, a borderline significant distortion was found in the Sib-TDT: 119 transmissions *v* 150 non-transmissions ($p = 0.057$; uncorrected p value).

Beside the positional candidate genes, we also looked in more detail at established candidate genes in the region. In the *AIRE* gene (21q22.3), we analysed two of almost 30 known mutations—R257X in exon 6 and the 13 base pair (bp) deletion in exon 8—which prevail in European populations.[20 21] These two mutations were screened in 96 T1D patients, but no carriers were identified. Fine mapping narrowed the linked region proximal to the *AIRE* gene, also suggesting no T1D association of this gene. By proteome analysis (that is, two dimensional gel electrophoresis in IL-1β exposed, diabetes-prone, Bio Breeding (BB-DP) rat islets of Langerhans in vitro), we showed that the protein carbonyl

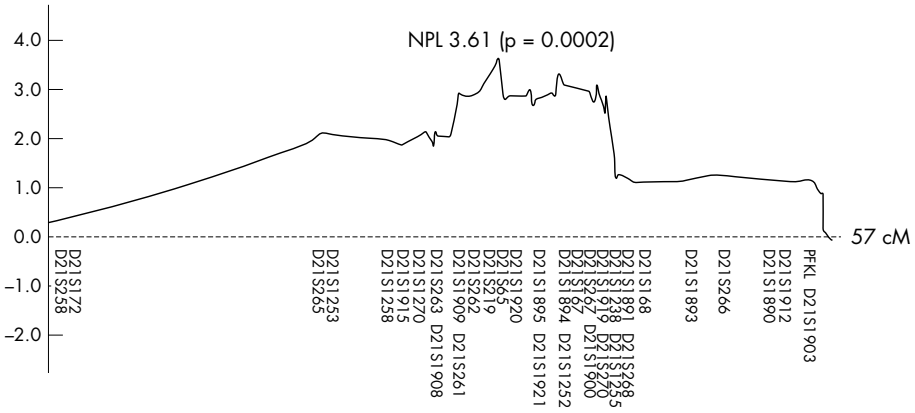

Figure 1 Multipoint non-parametric (NPL) score analysis. Peak maximum NPL score and corresponding p values, as well as approximate position of markers, are indicated.

reductase 1 (CBR1) encoded by the *CBR1* gene (21q22.12) was downregulated fivefold by IL-1β stimulation.[10] As IL-1β is believed to be the most important cytokine in the destruction of the β cells,[11] this finding may suggest a role for CBR1 in T1D, and makes the *CBR1* gene a functional candidate gene as well. Seven SNPs in coding regions of this gene—as well as two non-coding SNP—have been analysed (table 2); however, none of them demonstrated an association to T1D in the complete material.

Recent reports have described regulatory polymorphisms in RUNX1 binding sites in three other autoimmune diseases.[14–16] Four cSNPs in *RUNX1* (21q22.3) were analysed, as well as the intron 6 SNP, the C allele of which is reported to be associated with rheumatoid arthritis,[14] but no T1D association could be shown in the present study (table 2). For the intron 6 SNP (rs2268277), we observed 157 transmissions of the C allele *v* 174 transmissions of the G allele to affected offspring in the Sib-TDT test of the 253 T1D families. As the rheumatoid arthritis study was a case–control design, we also compared allele frequencies of 242 independent T1D patients with the allele frequencies among rheumatoid patients and controls[14] for the *RUNX1* intron 6 SNP. The T1D allele frequencies resembled the control population and not the distribution in the rheumatoid patients (data not shown).

Table 1 Multipoint non-parametric score analysis

Marker	NPL	p Value
D21S258	0.28	0.39
D21S172	0.32	0.38
D21S265	1.98	0.02
D21S1253	2.1	0.02
D21S1258	1.98	0.02
D21S1915	1.87	0.03
D21S1270	2.13	0.02
D21S263	1.87	0.03
D21S1908	2.06	0.02
D21S1909	2.05	0.02
D21S261	**2.92**	**0.002**
D21S262	**2.87**	**0.002**
D21S219	**2.96**	**0.002**
D21S1920	**3.61**	**0.0002**
D21S65	**2.86**	**0.002**
D21S1895	**2.85**	**0.002**
D21S1921	**2.78**	**0.003**
D21S1894	**2.99**	**0.001**
D21S1252	**3.12**	**0.0009**
D21S167	**2.92**	**0.0009**
D21S267	**3.05**	**0.001**
D21S1900	**2.55**	**0.005**
D21S1919	**2.68**	**0.004**
D21S1255	**2.6**	**0.005**
D21S270	**2.84**	**0.002**
D21S1238	1.64	0.05
D21S1891	1.48	0.07
D21S268	1.27	0.1
D21S168	1.1	0.14
D21S1893	1.11	0.13
D21S266	1.24	0.11
D21S1890	1.06	0.15
D21S1912	0.82	0.21
PFKL	0.13	0.45
D21S1903	−0.08	0.53

NPL scores and corresponding p values are shown. Markers conferring the 1 LOD drop support interval are in bold. NPL, non-parametric linkage.

In addition to testing for association in the complete material, we also carried out stratifications and analysis of subsets of data. Linkage has been demonstrated in the sibling pair families exclusively, and we hypothesised that the likelihood of detecting a susceptibility gene might be greater in families with several affected individuals than in simplex families. We therefore divided our sample into two subgroups, one containing the 155 sibling pair families and one containing the 98 simplex families. All genotyped SNPs were tested for association to T1D separately in the two groups. One SNP (rs2835655 in the *TTC3* gene), which showed borderline T1D association in the complete material ($p = 0.057$), was found to be significantly associated to T1D in the sibling pair family subgroup (76 *v* 103 transmissions, $p = 0.04$), whereas there was equal transmission (43 *v* 47 transmissions) in the simplex families.

In linkage analyses sibling pair families are exclusively informative and therefore have to be used. As it is likely that only a subset of the families will be responsible for the observed linkage signal, we divided the sibling pair families into two groups according to their linkage signal at the position corresponding to the peak NPL score of 3.61. All genotyped SNPs were then tested for T1D association, separately in the subgroup of families with sharing patterns consistent with linkage. For three SNPs, T1D association in the ''linked'' subgroup was demonstrated: for rs762178 in the *OLIG2* (oligodendrocyte lineage transcription factor 2) gene, 29 *v* 11 transmissions to affected individuals ($p = 0.01$) were observed, whereas 6 *v* 6 transmissions to unaffected offspring were found; for rs1805127 in the *KCNE1* (potassium channel, voltage gated, ISK related subfamily, member 1) gene, 19 *v* 39 transmissions to affected offspring ($p = 0.01$) and 7 *v* 9 transmissions to unaffected offspring were observed; and finally for rs20572 in the *CBR1* gene, 23 *v* 7 transmissions to affected offspring ($p = 0.047$) and only 2 *v* 2 transmissions to unaffected offspring were found. For none of these three

Table 2 Evaluation of 82 single nucleotide polymorphisms in positional candidate genes

	Bases	Amino acid	Material	Result	Gene
rs686364	T/C	Ser/Pro	253 families tested	No association	*CLDN8 (21q22.11)*
rs685967	A/G	Thr/Ala	253 families tested	No association	*CLDN8 (21q22.11)*
rs1557294	A/G	Thr/Ala	96 T1D screened	Not detected	*CLDN8 (21q22.11)*
rs762194	T/C	Gly/Gly	253 families tested	No association	*TIAM1 (21q22.1)*
rs2070417	G/T	Gly/Val	253 families tested	No association	*TIAM1 (21q22.1)*
rs2070418	G/A	Gly/Arg	253 families tested	No association	*TIAM1 (21q22.1)*
rs1804450	C/T	Thr/Ile	96 T1D screened	Not detected	*SOD1 (21q22.1)*
rs1804449	C/T	Asn/Asn	96 T1D screened	Not detected	*SOD1 (21q22.1)*
rs1804447	G/A	3′UTR	96 T1D screened	Not detected	*SOD1 (21q22.1)*
rs15012	A/G	3′UTR	96 T1D screened	Not detected	*SOD1 (21q22.1)*
rs1804448	A/C	3′UTR	96 T1D screened	Minor allele frequency <3%	*SOD1 (21q22.1)*
rs1050089	A/C	3′UTR	96 T1D screened	Minor allele frequency <3%	*SOD1 (21q22.1)*
rs2070371	T/C	Leu/Leu	253 families tested	No association	*HUNK (21q22)*
rs6517105	A/G	His/Arg	253 families tested	No association	*TCP10 (21q22.11)*
rs2017816	A/G	Lys/Lys	253 families tested	No association	*TCP10 (21q22.11)*
rs762178	A/G	Ser/Ser	253 families tested	No association	*OLIG2 (21q)*
rs1058857	C/T	Ser/Ser	96 T1D screened	Not detected	*IL10RB (21q22.1)*
rs2834167	A/G	Lys/Glu	253 families tested	No association	*IL10RB (21q22.1)*
rs1058859	C/A	Ala/Asp	96 T1D screened	Minor allele frequency <3%	*IL10RB (21q22.1)*
rs1058861	G/T	Ala/Ser	96 T1D screened	Minor allele frequency <3%	*IL10RB (21q22.1)*
rs2257167	G/C	Val/Leu	253 families tested	No association	*IFNAR1 (21q22.1)*
rs4986958	C/G	Thr/Arg	96 T1D screened	Not detected	*IFNGR2 (21q22.1–q22.2)*
rs1064579	T/G	Val/Gly	96 T1D screened	Minor allele frequency<3%	*IFNGR2 (21q22.1–q22.2)*
rs1802585	C/A	Asp/Glu	96 T1D screened	Not detected	*IFNGR2 (21q22.1–q22.2)*
rs2898199	A/T	Glu/Asp	96 T1D screened	Not detected	*GART (21q22.1)*
rs8971	A/G	Asp/Gly	253 families tested	No association	*GART (21q22.1)*
rs1804387	C/T	Leu/Phe	96 T1D screened	Not detected	*GART (21q22.1)*
rs2409496	G/C	Glu/Gln	Not typed		*GART (21q22.1)*
rs7280001	C/T	His/His	96 T1D screened	Not detected	*SON (21q22.1–22.2)*
rs7276682	A/C	Ser/Ser	96 T1D screened	Minor allele frequency<3%	*SON (21q22.1–22.2)*
rs1131912	C/T	Pro/Leu	96 T1D screened	Not detected	*SON (21q22.1–22.2)*
rs1051810	T/A	Asp/Glu	Not typed		*SON (21q22.1–22.2)*
rs1051811	T/A	Val/Glu	Not typed		*SON (21q22.1–22.2)*
rs7276194	T/C	Val/Val	96 T1D screened	Not detected	*ITSN1 (21q22.1–q22.2)*
rs2284567	C/T	Ala/Ala	253 families tested	No association	*ITSN1 (21q22.1–q22.2)*
rs2073370	T/C	Arg/Arg	253 families tested	No association	*ITSN1 (21q22.1–q22.2)*
rs2834286	C/T	His/His	Not typed		*ITSN1 (21q22.1–q22.2)*
rs2834296	A/G	Asn/Asn	253 families tested	No association	*ATP50 (21q22.1–q22.2)*
rs8129891	A/G	Thr/Ala	96 T1D screened	Not detected	*MRPS6 (21q21.3–q22.1)*
rs4817617	C/A	Gln/Lys	96 T1D screened	Not detected	*MRPS6 (21q21.3–q22.1)*
rs2234916	A/G	Thr/Ala	96 T1D screened	Minor allele frequency<3%	*KCNE2 (21q22.1)*
rs1805128	A/G	Asn/Asp	96 T1D screened	Not detected	*KCNE1(21q22.1–q22.2)*
rs1805127	A/G	Ser/Gly	253 families tested	No association	*KCNE1(21q22.1–q22.2)*
rs4252580	G/A	Pro/Pro	253 families tested	No association	*DSCR1 (21q22.1–q22.2)*
rs7280973	A/G	Gln/Gln	96 T1D screened	Not detected	*CLIC6 (21q22.12)*
rs6517254	C/T	Phe/Phe	253 families tested	No association	*CLIC6 (21q22.12)*
rs3171439	A/G	Asp/Gly	96 T1D screened	Not detected	*CLIC6 (21q22.12)*
rs1055309	A/C	Arg/Ser	96 T1D screened	Not detected	*RUNX1 (21q22.3)*
rs1055308	A/C	Arg/Ser	96 T1D screened	Not detected	*RUNX1 (21q22.3)*
rs1055307	C/G	Gly/Gly	253 families tested	No association	*RUNX1 (21q22.3)*
rs1055306	C/T	Gly/Gly	253 families tested	No association	*RUNX1 (21q22.3)*
rs2268277	C/G	*Intron 6*	253 families tested	No association	*RUNX1 (21q22.3)*
rs25678	C/G	Leu/Leu	253 families tested	No association	*CBR1 (21q22.12)*
rs1143663	G/A	cDNA/UTR	96 T1D screened	Not detected	*CBR1 (21q22.12)*
rs1051543	G/T	Val/Val	96 T1D screened	Minor allele frequency <3%	*CBR1 (21q22.12)*
rs2230191	G/A	Thr/Thr	96 T1D screened	Not detected	*CBR1 (21q22.12)*
rs20572	C/T	Ala/Ala	253 families tested	No association	*CBR1 (21q22.12)*
rs2230192	G/A	Val/Val	96 T1D screened	Minor allele frequency <3%	*CBR1 (21q22.12)*
rs1803321	A/G	3′UTR	96 T1D screened	Not detected	*CBR1 (21q22.12)*
rs5031013	A/C	Leu/Leu	96 T1D screened	Minor allele frequency <3%	*CBR1 (21q22.12)*
rs6413462	A/G	Ile/Val	96 T1D screened	Not detected	*CBR1 (21q22.12)*
rs2835284	A/G	Gln/Gln	96 T1D screened	Not detected	*CBR3 (21q22.2)*
rs881711	C/T	Asn/Asn	253 families tested	No association	*CBR3 (21q22.2)*
rs2835285	A/G	Val/Ile	96 T1D screened	Not detected	*CBR3 (21q22.2)*
rs881712	C/T	Val/Val	253 families tested	No association	*CBR3 (21q22.2)*
rs1056892	G/A	Val/Met	253 families tested	No association	*CBR3 (21q22.2)*
rs2236433	A/T	Ile/Ile	Not typed		*KIAA0136 (21q22.13)*
rs219779	G/A	Arg/Arg	96 T1D screened	Minor allele frequency <3%	*CLDN14 (21q22.3)*
rs2073416	A/G	Ser/Ser	253 families tested	No association	*SIM2 (21q22.2)*
rs2073601	A/C	Met/Leu	253 families tested	No association	*SIM2 (21q22.2)*
rs2845804	T/G	Intron	253 families tested	No association	*HLCS (21q22.13)*
rs219780	T/C	Thr/Thr	Not typed		*CLDN14 (21q22.3)*
rs1065759	C/T	Asn/Asn	96 T1D screened	Not detected	*HLCS (21q22.13)*
rs1065758	C/T	Ser/Ser	253 families tested	No association	*HLCS (21q22.13)*
rs2230182	C/T	Pro/Pro	253 families tested	No association	*HLCS (21q22.13)*
rs2276231	A/C	Arg/Ser	96 T1D screened	Minor allele frequency <3%	*DSCR5 (21q22.13)*
rs2154538	A/G	Val/Val	253 families tested	No association	*TTC3 (21q22.13)*
rs2835632	A/G	Lys/Lys	96 T1D screened	Not detected	*TTC3 (21q22.13)*
rs1053853	G/A	Lys/Lys	96 T1D screened	Not detected	*TTC3 (21q22.13)*

Table 2 Continued

	Bases	Amino acid	Material	Result	Gene
rs1053856	G/A	Lys/Lys	96 T1D screened	Not detected	*TTC3 (21q22.13)*
rs2835655	G/A	Lys/Lys	253 families tested	No association	*TTC3 (21q22.13)*
rs1053966	G/C	His/Asp	96 T1D screened	Minor allele frequency <3%	*TTC3 (21q22.13)*

cSNP, single nucleotide polymorphism in coding region; Not typed, no assay could be established; Not detected, no carriers of the polymorphism were detected. T1D, type 1 diabetes. In five of the 32 positional candidate genes no cSNPs existed in dbSNP; these are *CLDN17, IFNAR2, SYNJ1, CRYZL1*, and *OLIG1*.

SNPs could association in the ''unlinked'' subgroup of families be detected.

In order to evaluate the degree of linkage disequilibrium in the region, we assessed the pairwise LD indices between all microsatellite markers and coding SNPs, with a minor allele frequency of >3%, located in the ''one LOD drop'' support interval of 6.3 Mb (44 markers in all). Parental haplotypes were estimated by maximum likelihood estimates in GeneHunter version 1.2 and D′ values calculated in HaploXT (http://archimedes.well.ox.ac.uk/pise/haploxt-simple.html); the resulting pairwise D′ values are shown in table 3 (only marker sets with D′ values above 0.5 are given). Only a few small blocks with a threshold of D′ >0.5 were identified, including a block containing five consecutive markers (D21S267-D21S1900-rs2154538-rs2835655-D21S1919) and a block with three markers (rs881711-rs881712-rs1056892).

Haplotype testing

For all 35 microsatellite markers, we constructed haplotypes of three, four, and five adjacent microsatellite markers. The three and four marker haplotypes with a significantly distorted transmission (examined by TDT) to affected offspring are listed in tables 4 and 5. Only haplotypes transmitted or non-transmitted more than five times are listed. Haplotypes of five adjacent markers showed too few transmissions to be informative, as a maximum of only four transmissions for any of these five marker combinations was identified. Seven and 28 haplotypes of the four and three marker haplotypes, respectively, showed association to T1D. The location of these haplotypes, corresponding to the ''one LOD drop'' interval, is indicated by marker names in bold.

DISCUSSION

T1D is considered to be a complex genetic trait, with multiple genetic loci as well as environmental factors contributing to susceptibility. The genetics of T1D were initially studied by evaluating candidate genes for association to the disease, in either case–control or family based studies. The only consistent genes and regions showing significant T1D association have been the HLA region (6p21.3), the insulin gene region (11p15), and more recently *CTLA4* (2q33).[22] Several genome-wide linkage analyses in T1D have been undertaken, aiming at identifying the genetic determinants. Five complete genome scans,[3 23–26] a combined analysis of British and American genome scan data,[27] and some partial scans[28–32] have been done. In all, more than 20 genomic regions showing varying degrees of linkage to T1D have been identified. The aetiological mutation has not been determined for any of the identified T1D loci. Even in the HLA region several genes seem to be involved.[2] Most of the other putative loci comprise regions of up to 40 cM containing several genes (reviewed by Pociot and McDermott[2]). Mapping these genes is a major challenge, but it is important that identifications of linked regions in genome scans are followed by fine mapping in the same populations. Recently, the first identifications of disease-causing genes in complex diseases (Crohn's disease, asthma, and myocardial infarction) based on linkage analysis have been published, showing the feasibility of this approach.[33–36]

Based on the linkage results on chromosome 21 in the Scandinavian genome scan,[3] our current study provides an opportunity to identify genes of importance for T1D disposition on this chromosome in ethnically homogeneous populations such as the Danish and Scandinavian. Chromosome 21 is also interesting because an increased prevalence of T1D in Down's syndrome has been reported[37–39] and an earlier peak age at onset is suggested as well.[40] These observations, or some of them, could be a reflection of trisomy 21,[41] supporting the possible existence of T1D associated genes on this chromosome, and are probably also important in T1D in the general population. Furthermore, the existence of several functional candidate genes in the region makes it interesting.

The strategy used for the current project was to narrow the linked region, initially by linkage analysis including an increased number of microsatellites covering the region of

Table 3 Pairwise linkage disequilibrium values

Pairwise LD with consecutive markers, combinations with D′ >0.5			Additional (non-consecutive) pairwise combinations with D′ >0.5		
Marker 1	**Marker 2**	**D′ value**	**Marker 1**	**Marker 2**	**D′ value**
rs762194	rs2070417	0.359	rs2070418	rs4252580	0.689
rs2070417	rs2070418	0.964	rs2257167	rs1055307	0.68
rs6517105	rs2017816	0.527	rs2257167	rs1055306	0.601
rs8971	*D21S219*	0.581	rs8971	rs2284567	0.679
rs2073370	rs2834296	0.923	rs1055306	rs2230182	0.796
rs4252580	*D21S65*	0.577	rs881711	rs1056892	0.694
rs1055307	rs1055306	0.878	*D21S267*	rs2154538	0.676
rs25678	rs20572	0.89	*D21S267*	rs2835655	0.755
rs881711	rs881712	0.849	*D21S1900*	rs2835655	0.59
rs881712	rs1056892	0.661	*D21S1900*	*D21S1919*	0.514
rs2073416	rs2073601	0.816	rs2154538	*D21S1919*	0.656
rs2154538	rs2835655	0.9			
rs2835655	*D21S1919*	0.598			

Only combinations of any two markers with D′ values above 0.5 are shown.
LD, linkage disequilibrium.

Table 4 Three-marker haplotypes showing significant association to type 1 diabetes

Haplotype					
1	2	3	Transmissions	Non-transmissions	p Value
*D21S1258*10*	*D21S1915*4*	*D21S1270*9*	0	10	0.002
*D21S1258*10*	*D21S1915*4*	*D21S1270*16*	7	0	0.008
*D21S1915*4*	*D21S1270*8*	*D21S263*3*	5	0	0.025
*D21S1915*4*	*D21S1270*14*	*D21S263*3*	10	4	0.11
*D21S1915*4*	*D21S1270*17*	*D21S263*6*	0	5	0.025
*D21S1270*19*	*D21S263*7*	*D21S1908*5*	1	8	0.02
*D21S263*3*	*D21S1908*4*	*D21S1909*16*	18	8	0.049
*D21S1908*7*	*D21S1909*12*	***D21S261*3***	0	6	0.014
*D21S1909*12*	***D21S261*3***	***D21S262*9***	0	5	0.025
D21S261*2	***D21S262*9***	***D21S219*4***	13	3	0.012
D21S261*5	***D21S262*9***	***D21S219*6***	0	5	0.025
D21S262*8	***D21S219*6***	***D21S65*5***	0	5	0.025
D21S262*9	***D21S219*4***	***D21S65*6***	9	2	0.035
D21S262*9	***D21S219*4***	***D21S65*9***	13	4	0.029
D21S262*10	***D21S219*4***	***D21S65*9***	1	7	0.034
D21S262*10	***D21S219*6***	***D21S65*9***	1	8	0.02
D21S219*5	***D21S65*4***	***D21S1920*8***	8	0	0.005
D21S219*6	***D21S65*8***	***D21S1920*7***	5	0	0.025
D21S65*9	***D21S1920*7***	***D21S1895*9***	5	0	0.025
D21S1920*4	***D21S1895*3***	***D21S1921*8***	0	5	0.025
D21S1921*5	***D21S1894*4***	***D21S1252*5***	2	9	0.035
D21S1894*4	***D21S1252*10***	***D21S167*13***	3	11	0.033
D21S1894*5	***D21S1252*5***	***D21S167*13***	11	2	0.013
D21S267*4	***D21S1900*8***	***D21S1919*16***	1	8	0.02
D21S267*5	***D21S1900*8***	***D21S1919*10***	5	0	0.025
D21S267*5	***D21S1900*8***	***D21S1919*14***	0	6	0.014
D21S1900*8	***D21S1919*2***	***D21S270*8***	5	0	0.025
D21S1919*2	***D21S270*8***	*D21S1238*1*	5	0	0.025

Transmissions *v* non-transmissions of the haplotype in TDT test and the respective p values are given. Markers corresponding to the ''one LOD drop'' support interval are shown in bold.

interest in a multipoint NPL analysis. This strategy has been applicable since the chromosome was fully sequenced, though there is still some uncertainty about the exact map order of some of the markers. For the current study we initially used the Marshfield map (http://research.marshfieldclinic.org) for selection of microsatellite markers, but after advances in the completion of the human genome sequence, we used marker order and inter-marker distances based on the physical map of chromosome 21 (NCBI, National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) for further analyses. A recent study has shown the importance and impact of correct marker order for linkage analyses.[42]

By multipoint NPL analysis, a maximum NPL score of 3.61 was obtained at marker *D21S1920*, compared with a maximum LOD score of 2.33 (NPL score 2.64) in this population in the original study.[3] Based on a recent study of asthma susceptibility, it has been shown that the one-LOD decrease in the support interval of the linkage peak is likely to contain the susceptibility locus if the region has been saturated with markers.[35] In the present study the information content of this region was 95–100%. We therefore identified the corresponding support interval using NPL statistics (referred to as the ''one LOD drop'' interval) and found that it covered approximately 6.3 Mb, which was further studied by LD mapping with gene based SNPs.

The disease related LD was also assessed by a haplotype based association study. Of the four-marker haplotypes, seven were found to be significantly associated to T1D, six of which are located in the ''one LOD drop'' interval. Regarding three-marker haplotypes, 28 were shown to be associated to T1D, of which 21 were also located in the ''one LOD drop'' interval. That several haplotypes showed distorted transmission to diabetic offspring supports the existence of T1D susceptibility genes in this region.

We identified 32 candidate genes in this narrowed region. Several strategies can be chosen from here, ideally applying resequencing of all candidate genes in order to identify all mutations. In view of the still quite significant number, we chose to examine the genes by identifying SNPs in coding regions (cSNPs) of the genes, and initially to screen the identified 74 cSNPs in a panel of 96 diabetic subjects. SNPs with a minor allele frequency above 3% were further examined. These SNPs were genotyped in the complete

Table 5 Four-marker haplotypes showing significant association to type 1 diabetes

Haplotype						
1	2	3	4	Transmissions	Non-transmissions	p Value
*D21S1915*4*	*D21S1270*19*	*D21S263*7*	*D21S1908*5*	1	8	0.02
D21S262*10	***D21S219*4***	***D21S65*9***	***D21S1920*7***	0	7	0.008
D21S1921*5	***D21S1894*4***	***D21S1252*5***	***D21S167*13***	0	5	0.025
D21S1894*5	***D21S1252*5***	***D21S167*13***	***D21S267*4***	5	0	0.025
D21S267*11	***D21S1900*8***	***D21S1919*2***	***D21S270*8***	5	0	0.025
D21S1900*8	***D21S1919*2***	***D21S270*8***	*D21S1238*1*	5	0	0.025
D21S1919*2	***D21S270*7***	*D21S1238*1*	*D21S1255*7*	5	0	0.025

Transmissions *v* non-transmissions of the haplotype in TDT test and the respective p values are given. Markers corresponding to the ''one LOD drop'' support interval are shown in bold.

Danish T1D family material and examined for T1D association. However, none of the SNPs showed association to T1D, whereas one SNP in the *TTC3* gene did show distorted transmission to unaffected offspring, which might indicate a possible protective effect. The *TTC3* gene is expressed in most human tissues, including pancreas. It has been speculated that overexpression of this gene might be involved in some of the morphological anomalies observed in Down's syndrome.[43] We also undertook stratification of SNP data by dividing genotyping data in subgroups, according to simplex or sibling pair family status, and according to families carrying the linkage signal in the region and families not consistent with linkage, respectively. This approach is based on a theory predicting that genetically loaded individuals of families presumably provide a stronger signal and might facilitate identification of disease susceptibility variants.[44] This strategy of selecting families that show evidence for linkage to a region for further studies has been suggested and implemented previously.[35 45] Interestingly, we showed significant T1D association of a *TTC3* SNP rs2835655 in the sibling pair family subgroup, and additionally two other SNPs—rs762178 (*OLIG2*) and rs1805127 (*KCNE1*)—showed association in the subgroup of families responsible for the observed T1D linkage of the region. The degree of linkage disequilibrium in the linked region of 6.3 Mb was evaluated by pairwise LD measurements; however, we were only able to identify two blocks extending more than two markers with D′ values >0.5. Thus there do not appear to be large blocks with strong LD in this region, in agreement with previous studies on chromosome 21,[46 47] though one block containing five markers was demonstrated, spanning approximately 100 kb.

A few established candidate genes for autoimmune diseases are mapped to the region demonstrating linkage in the present study. The *AIRE* gene, originally considered a candidate gene of the linked region, was screened in 96 T1D patients for the two most common mutations causing the APECED syndrome in white populations, but no mutation carriers were identified. However, following the current fine mapping, the *AIRE* gene is no longer localised in the linked region but distal to this, and we did not continue with re-sequencing and further examinations of the gene. In support of this, others equally have been unable to identify any of these two mutations in T1D populations.[9 21 48] Furthermore, disomic homozygosity at the APECED locus has been proven not to be capable of explaining the increased level of autoimmunity in Down's syndrome.[49]

CBR-1 protein in rat islets is significantly downregulated following cytokine exposure[10] and is therefore likely to play a role in cytokine mediated β cell destruction. Typing seven cSNPs and two SNPs in 3′UTR of the *CBR1* gene did not show any significant association. However, in the ''linked'' family subgroup analysis, a significant association to T1D was found for the *CBR1* cSNP, rs20572 ($p = 0.047$).

Alloxan and streptozotocin (STZ) are pancreatic β cell selective toxins that have been used to probe the mechanisms underlying oxygen mediated damage to rodent β cells. Pretreatment with or concomittant administration of SOD1 confer protection against alloxan or STZ induced diabetes,[50 51] and cultured rodent islets can be protected from alloxan or STZ induced impairment.[51 52] Specific activity of pancreatic SOD1 has been shown to be significantly increased in alloxan resistant (ALR/Lt) compared with alloxan susceptible (ALS/Lt) mice,[12] and SOD1 transgenic mice are protected against oxidative stress.[53 54] In addition to two cSNPs, we also typed four SNPs of the 3′UTR. However, none of these showed association to T1D.

Indications that RUNX1 might be a common factor in autoimmunity have recently come from reports of disease associated mutations in *RUNX1* binding sites in three other autoimmune diseases.[14–16] In addition, association of the regulatory *PDCD1* variation originally found with SLE[16] has been replicated in T1D.[55] These reports make the *RUNX1* gene very interesting in T1D, substantiated by the location of the linkage peak we report here in exactly this region. RUNX1 protein is a DNA binding transcription factor with the context specific capability of activating or repressing gene expression.[56] We were not able to demonstrate any significant linkage of the known coding SNPs in this gene, nor of the intronic SNP in intron 6 of the *RUNX1* gene, which is associated to rheumatoid arthritis.[14] The allele showing association to rheumatoid arthritis in a dominant model was the C allele,[14] whereas in a family based study we were not able to show an increased transmission of the C allele to T1D affected offspring. On the other hand, we saw a slightly increased transmission of the G allele to affected offspring, though this was not significant. Allele frequency distribution for this SNP in T1D patients does not resemble that of the rheumatoid patients either. However, T1D association of borderline significance was observed in the ''linked'' family subgroup analysis for rs1055307 ($p = 0.055$). In addition, preliminary data from our group have shown that the mRNA expression of *AML1* (*RUNX1*) was significantly downregulated in a rat insulin producing cell line after exposure to IL-1 using Affymetrix chip analysis (Affymetrix Inc, Santa Clara, California, USA) (Nielsen K, personal communication), suggesting that *RUNX1* regulated gene expression may also be relevant in T1D pathogenesis.

Our data suggest that one or several T1D linked genes exists in the region, even though no evidence for association was found for the tested SNPs in the complete material. Despite evidence for linkage in the Danish population, linkage to T1D on chromosome 21 has not been reported in other genome scans. However, it cannot be ruled out that it was missed in other studies owing to the inclusion of relatively few markers of this region on chromosome 21. It is estimated that the information content of this particular region in previous genome scans did not exceed around 0.66. Certainly further genotyping and refinement of the genetic map corresponding to this region on chromosome 21 is important. Our data have so far not been replicated in other populations as our strategy was initially to fine map the linked region in the same populations in which linkage was demonstrated, in order to localise a specific gene and show aetiological or otherwise significant mutations, before replicating the findings in other populations.

LD mapping with SNPs coupled with genome scans is being used increasingly and is considered valuable for fine mapping and genetic association studies.[57 58] Genotyping this number of SNPs is still time consuming and costly, but important in terms of fully characterising and fine mapping this region on chromosome 21. Identification of a disease associated gene based on linkage analysis has so far never been obtained successfully in T1D.

Our study reduced the region linked to T1D from more than 20 Mb to 6.3 Mb and increased the NPL score from 2.64 to 3.61. Furthermore, the number of possible susceptibility genes was reduced to 32. Although we were not able to identify a single gene variant responsible for the observed linkage, we demonstrated significant support for several combinations of markers within this region, and some evidence for association to T1D of variants of the *TTC3*, *OLIG2*, *KCNE1*, and *CBR1* genes in conditioned analyses.

As our data strongly suggest the existence of T1D linked genes in the region, further characterisation of the candidate genes on chromosome 21 is very important, implying analyses of regulatory and promoter regions, in which variants of potential importance in regulation of gene expression and thereby functional significance are likely to

be located. The possible significance of non-coding SNPs in disease predisposition have been demonstrated several times (for example, *CALP10*, *CTLA4*, and *RUNX1*).[14–16 22 45]

The identification of genes responsible for disease will lead to a better understanding of the mechanisms underlying the disease process and thereby perhaps in the future be helpful specifically in developing intervention against T1D.

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