Brief Genetics Report

IL-12p40 Is Associated With Type 1 Diabetes in Caucasian-American Families

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The *IL-12p40* locus has recently been shown to be associated with type 1 diabetes (1). Here, we report the identification of novel microsatellite and single-nucleotide polymorphisms (SNPs) within the *IL-12p40* gene and a significant association between a (ATT)n repeat marker and type 1 diabetes in 364 U.S. Caucasian sib-pair families ($P < 0.006$). Haplotype analysis using the (ATT)n repeat (*D5S2941*) and the C1159A SNP at the 3′ untranslated region of *IL-12p40* showed a significant association ($P = 0.02$). Expression studies in individuals heterozygous for the C1159A SNP indicated that the expression of the 1159A allele is ~50% higher than that of the 1159C allele. These results provide genetic and functional evidence for *IL-12p40* as a type 1 diabetes susceptibility gene. *Diabetes* 51:2334–2336, 2002

A number of susceptibility genes have been mapped to chromosomal regions in both mouse and humans using genome-scanning techniques (2–7). However, identification of the specific genes has been difficult because the contribution of each gene is usually very small. The analysis of candidate genes has been a very fruitful approach in the studies of type 1 diabetes susceptibility genes. For example, the first two type 1 diabetes genes, *type 1 diabetes1* (HLA) and *type 1 diabetes2* (*INS*), were both identified by the candidate gene approach. Recently, an association between type 1 diabetes and *IL-12p40* was reported (1). Interleukin (IL)-12 is known to induce Th1-cell differentiation and cytokine production (8). A link between type 1 diabetes and IL12 is also suggested in NOD mice, BB rats, and humans (9–17). There is also a correlation between the expression of IL-12 and the destruction of insulin-producing cells in the course of disease progression in NOD mice. Administration of IL-12 to young NOD mice accelerates diabetes, which correlates with a higher production of interferon-γ (IFN-γ) and a lower production of IL-4 (9,18–19).

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BAC, bacterial artificial chromosome; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharides; SNP, single-nucleotide polymorphism; TDT, transmission disequilibrium test; UTR, untranslated region.

We evaluated the candidacy of *IL-12p40* as a type 1 diabetes susceptibility gene by association studies with polymorphic markers within *IL-12p40*. Direct DNA sequencing of PCR products amplified from DNA pools of patients and control subjects revealed a C-to-A polymorphism at position 1,159 of *IL-12p40* (GenBank Accession no. NM_002187). This single-nucleotide polymorphism (SNP) was designated as C1159A, and the mutation creates a new *Taq*-1 restriction site that is convenient for genotyping. This is the same SNP reported in a recent study (1). Because genomic sequence for *IL-12p40* was not available at the time of the study, we determined the partial genomic sequence of the gene from a bacterial artificial chromosome (BAC) clone screened from the Research Genetics BAC library. Three microsatellite markers were identified from the partial genomic sequences, namely (ATT)n, (CT)n, and (AT)n(CA)n. Analyses of these three markers in 200 diabetic families indicated that the (ATT)n microsatellite was polymorphic and that the other two repeats had very limited polymorphism. The (ATT)n marker is designated as *D5S2941*. We observed two alleles at this locus. In the study population, allele 1 with eight repeat units has a frequency of 83.5%, and allele 2 with nine repeat units has a frequency of 16.5%. We also identified intronic insertion of the G nucleotide in the intron immediately upstream of the 3 untranslated region (UTR) (position 214 of GeneBank AY064126). This insertion polymorphism is in complete linkage disequilibrium with the C1159A SNP.

We evaluated the association between type 1 diabetes and *IL-12p40* polymorphisms in 364 U.S. Caucasian affected sib-pair families in the HBDI (Human Biological Database Interchange) collection using a transmission disequilibrium test. The 1159A allele was transmitted more often to affected individuals (Table 1), although it did not reach statistical significance ($P = 0.08$). The allele 2 of *D5S2941* is transmitted more often to affected individuals ($P = 0.006$) (Table 1). Haplotypes were constructed for the two markers using family data, and extended transmission disequilibrium test (TDT) showed significant association ($P = 0.02$). We did not find any significant difference in HLA-DR subgroups (data not shown).

To demonstrate the functional significance of the observed association, we examined the relative expression levels of the *IL-12p40* alleles in three heterozygous individuals for the C1159A SNP located at the 3UTR. Total RNA was extracted from peripheral blood lymphocytes after a 4-h induction with lipopolysaccharides (LPS). After

TABLE 1
Transmission disequilibrium test with *IL12p40* markers

Markers	Allele	Frequency	T (%)	NT (%)	*P*
C1159A	A	15%	150	122	0.08
D5S2941	2	16.5%	208	156	0.006
Haplotype (ETDT)					0.02

T, transmitted; NT, not transmitted.

RT-PCR, the amplified PCR products were digested with *Taq*-1 and resolved on a 1.5% agarose gel. The band intensities for the undigested allele (1159A) and digested allele (1159C) were measured by an optic documentation system. The background-corrected data suggest that the expression level of the 1159A allele is ~50% higher than that of the 1159C allele (Fig. 1). These results suggest that a regulatory mutation(s) in linkage disequilibrium with the C1159A SNP may be responsible for the allelic difference of *IL-12p40* expression.

Our observation is consistent with the correlation between mRNA expression of IL12 and β-cell destruction in NOD mice (20) and the acceleration of diabetes by IL-12 in young NOD mice (15). Functionally, the increased expression level of the *IL-12p40* 1159 allele is likely a risk factor for type 1 diabetes. Genetically, the transmission of the 1159A allele is increased in comparison to random chance in our own study and in a previous report (1). Therefore, *IL-12p40* is likely a susceptibility gene for type 1 diabetes. However, the specific etiological mutation in the *IL-12p40* gene has not yet been identified. Our functional studies suggest that the mutation(s) is most likely located in the regulatory region of the *IL-12p40* gene.

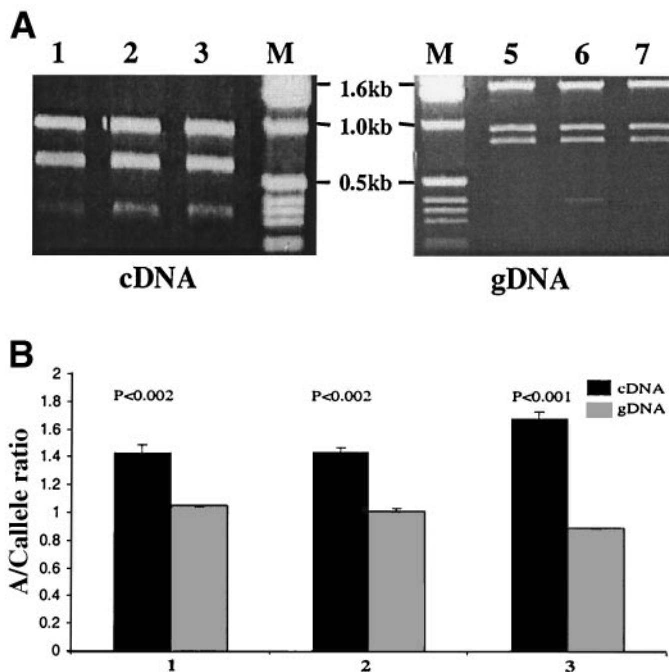

FIG. 1. Expression analysis of the *IL-12p40* gene in heterozygous individuals. *A*: Fragment of 844 and 1682 bp containing the C1159A SNP was amplified from cDNA and genomic DNA of three heterozygous individuals, respectively. The PCR products were digested by *Taq*-1 and electrophoresed on 1.5% agarose gels. The digested 1159C allele yields two fragments of 549 and 295 bp from cDNA and 906 and 776 bp from genomic DNA. *B*: Ratio of 1159A vs. 1159C allele in cDNA and gDNA. The intensities and background for each band were measured using the Eagle Eye documentation system. The total intensity for the digested 1159C allele was derived from the two digested bands. The mean and SDs from three replicates are presented in the chart. The difference of ratios between cDNA and genomic DNA was tested using the Student's *t* test. These results suggest that the 1159A allele is expressed at a higher level than the 1159C allele.

RESEARCH DESIGN AND METHODS

Screening of polymorphisms. A human BAC library from Research Genetics was screened for clones containing the *IL-12p40* gene by PCR. BAC DNA was purified using the Qiagen 12125 plasmid Mini Kit according to the manufacturer's instructions. Primers complimentary to cDNA sequences of *IL-12p40* were designed and used for amplifying genomic sequences from the BAC clone positive for *IL-12p40*. Amplified PCR products were purified and directly sequenced using a previously described method (21,22). Briefly, 50–100 μl PCR products were electrophoresed in 1.5% agarose gel, and expected bands were excised and transferred into 1.5 ml Eppendorf tubes. The tubes were kept at −80°C for 30 min and were then transferred to room temperature. The agarose gels were smashed and kept again at −80°C for 10–15 min. PCR products were eluted out of the gel by pipetting at room temperature. The eluted DNA can be used as sequencing template. Each sequencing reaction (20 μl) contains 20 ng of PCR product, 2 μl Big Dye reaction mix (PerkinElmer), 1 μl sequencing primer (3.5 pmol), and 3.5 μl 5× sequencing buffer (400 mmol/l Tris-Hcl and 10 mmol/l MgCl2, pH 9.0). Sequencing reactions were carried out for 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The products were purified using 0.1 vol 3 mol/l sodium acetate (pH 5.2) and 2.5 vol absolute ethanol. The tubes were left at room temperature for 10 min and were then centrifuged for 20 min at 15,000 rpm at bench top centrifuge. The DNA pellet was washed once with 250 μl 70% ethanol and then dried in a vacuum drier for 10 min. The dried DNA was dissolved in 20 μl template suppression buffer (Perkin Elmer). The sequences were determined with an ABI 310 or ABI377 automated DNA sequencer.

Genotyping. The microsatellite marker (ATT)n was genotyped using the forward primer IL12(ATT)-F (5′-GAGGAGTCAGCCAACTGTA) and reverse primer IL12(ATT)-R (5′GACTAAGCTCTACTGGACTG). The forward primer was end labeled with ^{32}p (dATP) using polynucleotide kinase at 37°C. PCR amplifications were performed with 20 ng genomic DNA in 12 μl reaction volume containing 5 mmol/l KCl, 10 mmol/l Tris-Cl, pH 8.3, 1.5 mmol/l MgCl2, and 60 μmol/l of each dNTP. Samples were initially denatured at 94°C for 2 min followed by 32 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 30s and an additional extension at 72°C for 2 min. Amplified products were mixed with 2 vol loading mix (90% vol/vol formamide, 10 mmol/l EDTA, pH 8.0, 0.3% bromophenol blue, and 0.3% xylene cyanol), denatured at 95°C for 5 min, and then cooled on ice while loading. An aliquot of 2–4 μl of PCR products was loaded on a 6% sequencing gel. The gels were exposed to film, kept at −80°C for 8–12 h, and developed.

A 1046-bp fragment containing the C1159A SNP at the 3UTR of *IL12-p40* was amplified using the forward primer IL12p40–1 (5′-ATTTGGAGGAAAAGT GGAAGA) and the reverse primer IL12p40–2 (5′- AATTTCATGTCCTTAGCC ATA). Amplified products (12 μl) were digested using 10 units of *Taq*-1 (Gibco) per reaction for 4 h at 65°C and then for 10 min at 95°C. Digested products were electrophoresed on a 2.5% agarose gel. DNA fragments were visualized by ethidium bromide. The 1159A allele yields two fragments, 906bp and 140bp, respectively.

Expression analysis. Peripheral lymphocytes were separated by Ficoll-Hypaque gradient and then stimulated with LPS (Sigma) (1 μg/ml) for 4 h. Total RNA was extracted using Trizol (Sigma). First-strand cDNA was made by reverse transcriptase (Gibco) according to the manufacturer's instructions in a total volume of 25 μl. The cDNA was diluted to 1:2, 1:4, and 1:8 in TE buffer (pH 8.00), and 2 μl of the diluted cDNA was used for 30 cycles of PCR amplification in triplicates using forward primer IL12F3 (5′-CCCAGCTGCTG AGGAGAGTC) and reverse primer IL12R3 (5′CAGGACCGCTACTATAGCT CA), which generate a 844-bp fragment containing the C1159A polymorphism. Amplification from genomic DNA using the same primers generates a frag-

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ment of 1682 bp. The amplified PCR products were digested with *Taq*-1 and resolved on a 1.5% agarose gel. Band intensities and background were measured by Eagle Eyes documentation system (Stratagene).

Statistical analysis. The TDT was used to assess association. The χ^2 test was used to evaluate the deviation from 50% of the expection of transmission from heterozygous parents to affected siblings. Extended TDT was used for markers with more than two alleles using a logistic regression analysis (23).

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GenBank accession nos. are AY046592 and AY046593 for SNP C1159A and AY046594 for *D5S2941*.

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