No Association Between *lck* Gene Polymorphisms and Protein Level in Type 1 Diabetes

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We previously described a reduced expression of the protein tyrosine kinase Lck in T-cells from type 1 diabetic patients, the origin of which is still unknown. The human *lck* gene, located on chromosome 1p35-34.3, was evaluated as a candidate susceptibility gene for type 1 diabetes. A molecular scan of the sequence variations in the coding, the relevant promoter, and most of the intronic sequences of the *lck* gene (representing a total of 10.5 kb fragment) was performed in 187 Caucasian subjects including 91 type 1 diabetic patients and 96 normoglycemic control subjects. We identified 35 sequence variations, including one deletion and 34 single nucleotide polymorphisms (SNPs), 33 of them being new. Four variants were frequent but not significantly associated with diabetes or Lck protein level. Of the SNP variants, 11 were only found within the diabetic population and some were associated with low Lck protein levels. The low frequency of these polymorphisms did not permit any statistically significant correlations with the disease status, suggesting that the *lck* gene probably does not contribute to genetic susceptibility to type 1 diabetes. *Diabetes* 51:3326–3330, 2002

Genetic susceptibility to type 1 diabetes reflects the combined effect of multiple polymorphic genes interacting with environmental events (1). Recent genome-wide linkage analysis has shown that at least 18 loci outside the HLA class II region are candidate regions involved in susceptibility to type 1 diabetes; some of these loci contain genes with immunological functions. Disease results from the destruction of the insulin-producing β-cells in the islets of Langerhans by autoreactive lymphocytes. The breakdown of self-tolerance to islet antigens could be attributed in part to defective T-cell activation (2). Both in human and in the NOD mouse model, peripheral T-cells are characterized by functional defects after T-cell receptor (TCR)-mediated lymphocyte activation, including a decreased proliferative response (3,4) and a biased T-helper (Th)-1 and Th2 cytokine profile (5,6). In type 1 diabetic patients, the hyporesponsiveness of T-cells correlates with the low expression of Lck, a lymphoid protein tyrosine kinase of the Src family (7). In the NOD mouse, a diminished recruitment of CD4-associated Lck to the TCR has been documented (8), and one candidate region, *idd9.1* in chromosome 4 encompasses the *lck* gene (9). Lck regulates early activation events after TCR engagement (10), thymic differentiation (11), Th1/Th2 differentiation (12), apoptosis (13), and homeostatic proliferation of naïve T-cells (14). The human *lck* gene, located on chromosome 1q35-34.3, has 12 exons distributed across ~14 kb of genomic DNA. Its expression is driven by two promoters (distal and proximal) active at different stages of development and separated by at least 35 kb (15). The lower protein expression in humans and the existence of a susceptibility region containing *lck* in the mouse model prompted us to test whether *lck* could be a new susceptibility gene for type 1 diabetes. A systematic survey of the natural sequence variation in the coding, the relevant promoter, and most of the intronic sequences of the *lck* gene was performed by direct sequencing in a cohort of healthy subjects ($n = 96$) and type 1 diabetic patients ($n = 91$). Then, the nature, frequency, and association of variants were analyzed in the two groups and correlated to the Lck protein level.

RESEARCH DESIGN AND METHODS

Subjects. Genomic DNA was prepared from purified mononuclear cells from 91 randomly recruited French Caucasian type 1 diabetic patients, diagnosed according to the American Diabetes Association classification criteria (16), the study population consisted of 43 males and 48 females (mean age 41 ± 11 years, mean HbA_{1c} 8.16 ± 1, mean age at diagnosis 22 ± 11 years). Controls consisted of stored DNA samples derived from 96 healthy volunteers without any familial history of autoimmunity. An independent group of 10 control subjects was recruited for the biochemical analysis. For the second screening, genomic DNA from 53 type 1 diabetic patients (27 males, 26 females, mean age 44 ± 16 years) and 34 control subjects (17 males, 17 females, mean age 45 ± 18 years) was analyzed. Ethic approval was obtained from the local committee (CCPPRB Marseille 2).

PCR amplification, sequencing, and variant analysis. Sequences of the overlapping primers sets used for PCR amplification and sequencing are given in Appendix 1 (http://diabetes.diabetesjournals.org). The target regions were amplified from each genomic DNA sample in seven segments (S1 to S7, Fig. 1*A*). PCRs were performed on 40 ng of genomic DNA using ExTaq polymerase (Takara, Gennevilliers, France). Cycling conditions included an initial denaturation at 94°C for 5 min followed by 35 cycles at 98°C for 5 s, 30 s at various temperatures according to the primers, 68° for 5 min, and a final extension step at 72° for 10 min. The purified PCR products (2 μl) were sequenced in both directions by means of an ABI PRISM dGTP Big Dye Terminator on a 3700 automatic sequencer (PE Applied Biosystems, Courtaboeuf, France).

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Additional information for this article can be found in online appendixes at http://diabetes.diabetesjournals.org.

SNP, single nucleotide polymorphism; TCR, T-cell receptor; Th, T-helper; TNF, tumor necrosis factor.

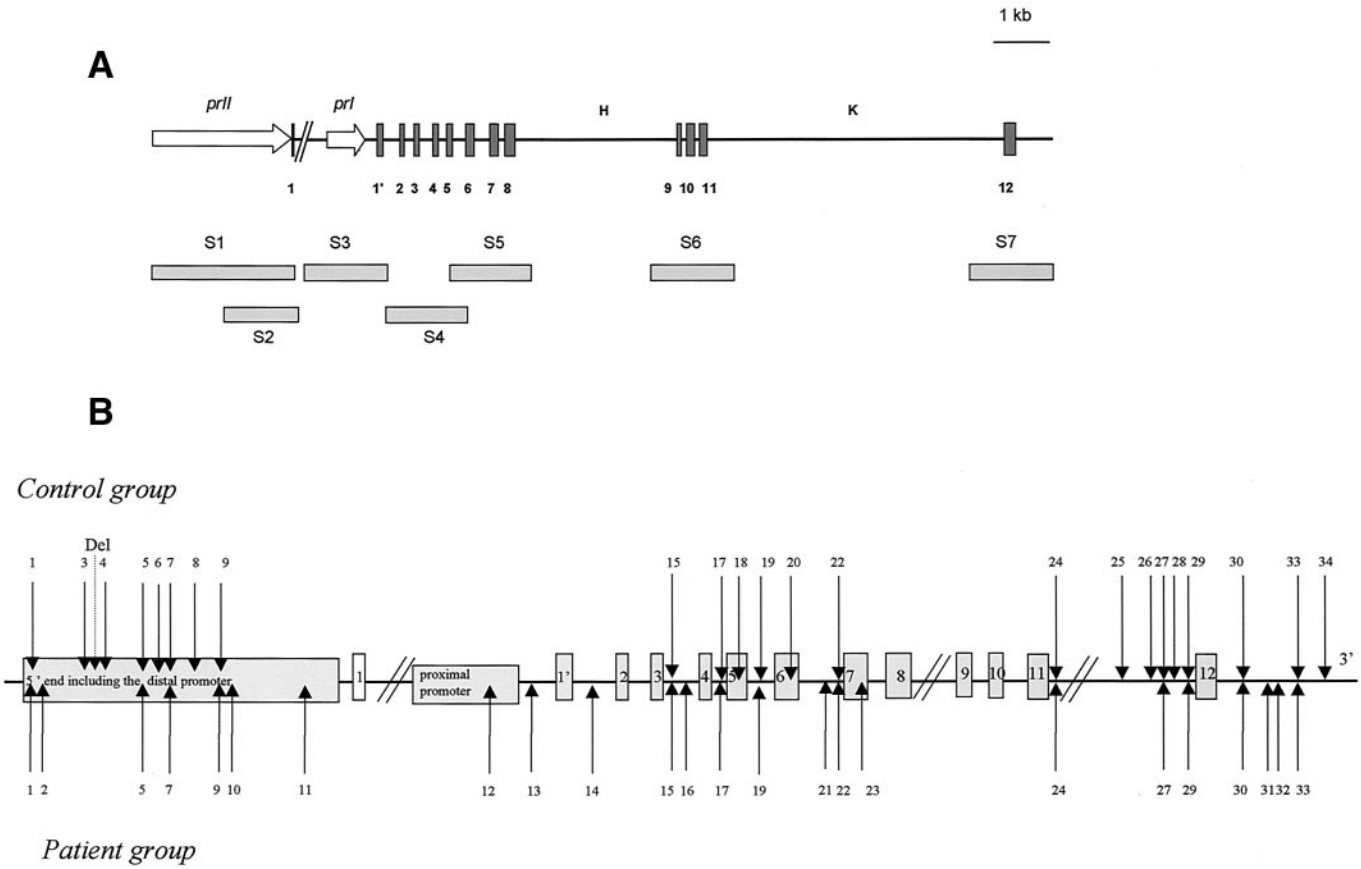

FIG. 1. *A*: Genomic organization of the *lck* gene. The two promoters are indicated by white arrows. The gray horizontal bars represent the different stretch of the *lck* gene that has been sequenced. The total sequenced region spans 10.5 kb. *B*: Location of the deletion (dotted arrow) and SNPs (continuous arrow) in the control and patient groups. Polymorphisms identified in the control and diabetic groups are represented respectively in the upper and lower part of the figure.

Lck protein expression. Lck protein levels were determined by a Western blot in resting T-cells from the diabetic cohort ($n = 91$) and matched healthy subjects ($n = 10$). Procedures for peripheral mononuclear cell extraction, cell lysate preparation, and protein detection and quantification by densitometry have already been described (7).

Statistical analysis and nucleotide diversity/heterozygosity calculation. Data from patient and control groups were compared using the Student's t and χ^2 tests. Variant frequency (V_f) was calculated using $V_f = (2x + y)/2z$, where x is the number of homozygous carriers of the rare allele, y is the number of heterozygous carriers, and z is the number of homozygous carriers for the common allele.

RESULTS AND DISCUSSION

Nature and distribution of the sequence variations in the *lck* gene. We generated a contiguous baseline reference for the *lck* gene sequence (17,484 nucleotides, GenBank accession no. BN000073) by assembling existing exon, intron, and regulatory sequences obtained from the EMBL Nucleotide Sequence Database and the Ensembl Genome Server (http://www.ensembl.org), included in contigs AC022307.14.115592.135590 and AC022307.14.94802.115491. For each subject, a 10520 nucleotide fragment (60% of the reconstituted sequence) was sequenced, covering part of the 5′regulatory region containing two promoters, all exons, all intron/exon boundaries, all short introns, part of the two large introns H and K, and the 3′ untranslated region. The location of the sequenced fragments is represented in Fig. 1*A*.

Comparison of the reference sequence with that obtained for 187 subjects (91 patients and 96 control subjects) allowed the identification of 35 sequence variations (Fig. 1*B*). We found 34 SNPs and only an AC deletion in the 5′ end of the gene in one control subject. Comparison with the previously described SNPs (http://snp.cshl.org and http://www.ncbi.nlm.nih.gov/SNP/) showed that 33 variants were new; the SNP 30 has been previously described in the public database. All variable sites were biallelic and 33 were synonymous SNPs. Transition substitutions were more prevalent (22 of 34, 64.7%) than transversions. Most variable sites (31 of 34, 91.1%) were found in noncoding regions, in agreement with the predicted rare SNP diversity in human genes (17,18). Some SNPs were close to exon boundaries but not within consensus splicing sites.

***lck* gene SNPs in control subjects and patients.** Around half of the individuals had no variation in the scanned region of the *lck* gene. SNP characteristics for each group are summarized in Table 1. The frequency of variant carriers was not statistically different in the two groups (46% in the control group and 44% in the diabetic group, $\chi^2 = 0.07$). Among the SNP carriers, most of them had only one or two SNPs, and only three control subjects and one patient were homozygous carriers of the most common variants (SNP 7 or SNP 30) (Fig. 2*A* and *B*, black boxes).

In the control group, 21 of 23 variable sites were in noncoding regions. The two SNPs in coding regions were located in exons 5 and 6, inducing a silent mutation (E414E in exon 5, third base mutation, SNP 18) and a

TABLE 1
SNP characteristics for each group

SNP, no.	Nucleotide variation	Position	Location	Comments	Minor allele frequency ($\times 10^{-2}$)	
					Control subjects	Patients
1	A to G	173	5′ end		5.36	2.41
2	G to A	231	5′ end		0	0.56
3	G to C	980	5′ end		3.45	0.56
—	AC deletion	1135–36	5′ end		0.52	0
4	C to A	1159	5′ end		0.53	0
5	C to T	2045	5′ end		0.60	1.23
6	G to A	2295	5′ end		3.05	0
7	G to T	2615	5′ end		20	22.73
8	A to G	2717	5′ end		0.57	0
9	G to A	2795	5′ end		1.81	5.36
10	G to A	2822	5′ end		ND	1.64
11	A to G	3521	5′ end		0	0.82
12	A to C	4849	prox pr		0	0.58
13	C to T	5268	5′ UTR		0	0.56
14	G to A	5770	intron A		0	0.61
15	C to A	6298	intron C		0.53	0.57
16	C to T	6338	intron C		0	0.57
17	G to T	6656	intron D		0.53	0.53
18	GAG to GAA	6703	exon 6	silent E	0.53	0
19	C to G	6949	intron E		0.53	0.53
20	CGG to TGG	7032	exon 6	R to W	0.53	0
21	A to G	7293	intron F		0	0.53
22	G to T	7357	intron F		1.06	1.08
23	CCG to CCC	7499	exon 7	silent P	0	0.56
24	T to C	11425	intron K		0.54	1.19
25	T to C	16028	intron K		0.60	ND
26	C to T	16285	intron K		0.56	0
27	C to G	16294	intron K		0.56	0.57
28	T to A	16401	intron K		0.53	0
29	G to A	16492	intron K		0.53	0.56
30	A to G	17010	3′ UTR		10.63	10.67
31	A to G	17027	3′ UTR		0	0.56
32	A to T	17110	3′ UTR		0	0.56
33	T to C	17237	3′ UTR		0.54	1.12
34	C to T	17386	3′ end		0.54	0

ND, not determined; UTR, untranslated region.

nonsynonymous substitution leading to a basic hydrophilic to an apolar hydrophobic amino acid change in the protein sequence (R502W in exon 6, SNP 20), respectively. In the patient group, we found 24 variants, including one silent third base substitution (P696P, SNP 23) in exon 7. The SNPs were differentially distributed among the two groups (Fig. 1*B*). Ten SNPs were specific to the control group and 11 to the diabetic group. Among them, six (SNP 2, SNP 10–14) were located in the 5′region of the gene, including both promoters, but none mapped in identified transcriptional regulatory elements.

SNP frequencies and combinations in the two groups. Most variations were rare (<5%) in both groups (Table 1). Among them, SNP 3 and SNP 6 were more frequent in the control group but these differences were not statistically significant after analysis in the two new cohorts of patients ($n = 53$) and control subjects ($n = 37$). Lck polymorphisms (>5% variation) were identified in four positions corresponding to SNP 1, 7, 9, and 30, but the frequency between groups was not statistically different. We then determined whether a combination of polymorphisms could characterize each group irrespectively of their allelic distribution. We identified 20 distinct variant combinations, 12 in the control group and 10 in the patient group (arrows in Fig. 2*A* and *B*); only two of them (SNPs 7–30 and SNPs 1–9) were shared by the two groups. Most of the SNP combinations were only represented once. All *lck* variations and polymorphisms (SNP 1, 7, 9, and 30) were not statistically associated with disease status when considered either isolated or in combination, but their frequency and the limited cohort size did not allow an extensive analysis.

SNPs and Lck protein expression in diabetic patients. We then examined the individual contribution and the combination of the effects of variants on the Lck protein level (Fig. 2*B*). As previously reported (7), we found at least a twofold reduction in the amount of basal detergent-extractable Lck for 64% of the patients. In some patients, the protein level was very low and associated with unique variants in the diabetic population (for example, SNP 31 was present in patient no. 7, whose Lck level was 4%). Similarly, some combined SNPs were associated with low protein level. Among the 10 SNP associations found in patients, 2 fell in this category (SNPs 1–7 in patient no. 3, Lck 0%; or SNPs 12–14 in patient no. 9, Lck 6%) but were only represented once. However, the rare

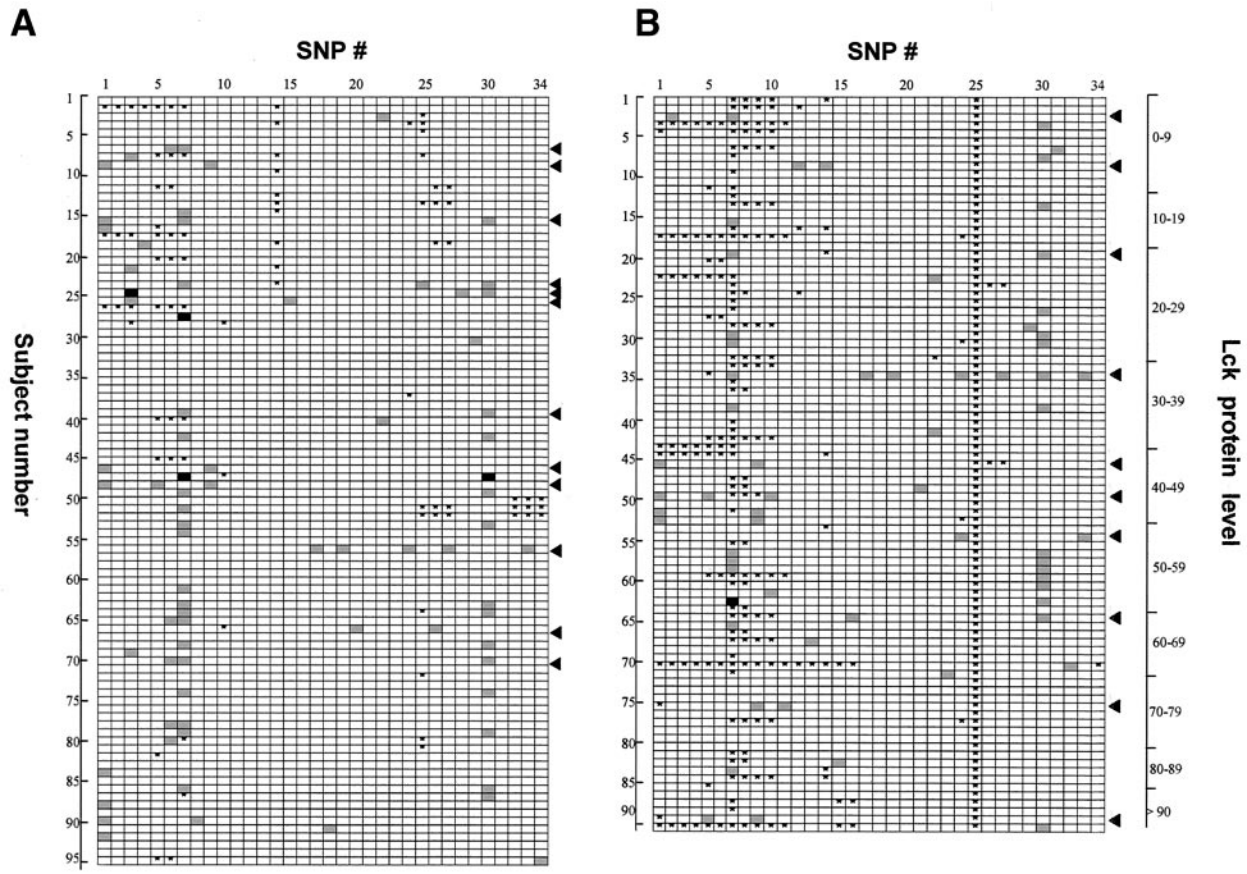

FIG. 2. *A* and *B*: Genotype and SNP combinations of the control subjects and type 1 diabetic patients. Individual sample identifiers are shown on the left of the array and variants are numbered on the *x*-axis consecutively across the top. At each site, all individuals homozygous for the common allele are represented in white, heterozygous in gray, and homozygous for the rare allele in black. The stars denote positions where genotypes were not obtained. In the diabetic group (panel *B*), Lck genotype is confronted with protein expression level.

occurrence of these situations prevented the establishment of any functional correlation between the two parameters. For each SNP, the mean Lck protein level for each genotype is given in Appendix 2 (http://diabetes.diabetesjournals.org).

Based on the hypothesis that common genetic variants underlie susceptibility to common disease, we finally evaluated whether the presence of the polymorphisms found with the higher frequency (SNPs 1, 7, 9, and 30) could be associated with low Lck protein level (Fig. 3). For these four SNPs, no difference was observed between the heterozygous carriers of the rare allele and the homozygous carriers of the common allele.

In conclusion, a systematic survey on a larger cohort of subjects and family studies is required to evaluate the functional relevance of all SNPs apparently specific, alone or in combination, of patients. Nevertheless, no obvious linkage between individual or combined variants and the protein level was observed in the diabetic group. We cannot exclude the presence of relevant SNPs outside of

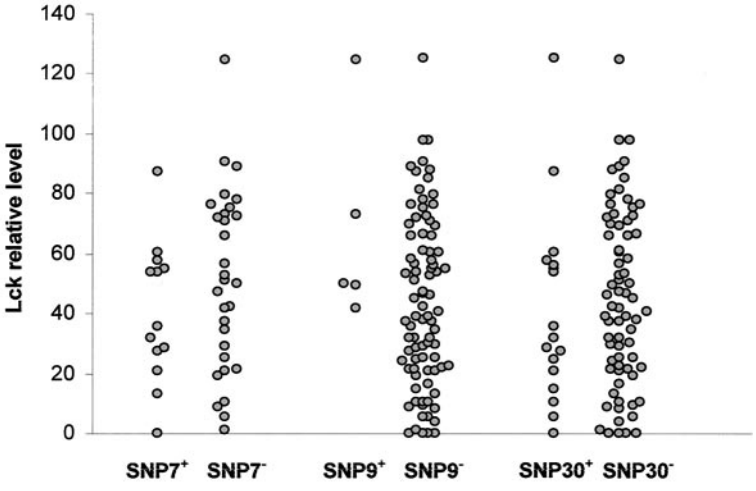

FIG. 3: Genotype/phenotype relationships among frequent polymorphic variations in the *lck* gene in diabetic patients.

the scanned region, especially in the regulatory regions. Nevertheless, the mechanisms responsible for the Lck protein alterations in T-cells from most of the patients remain unclear and could be related to an increased turnover of the protein (19), to a downregulation by soluble factors such as the tumor necrosis factor (TNF) (20), by the oxidative stress (21), or to a deficiency in a protein or transcription factor controlling Lck synthesis or stability. While we found no overall evidence for linkage of *lck* polymorphisms to type 1 diabetes, our study is the first analysis of the *lck* variant genotype distribution in a population of Caucasian subjects (n = 187), and the description of the variants in this gene could be relevant for the understanding of genetic susceptibility to the numerous other autoimmune disorders in which the Lck protein is downregulated (22–25).

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