

Complete mutation scan of the human Fas ligand gene: Linkage studies in Type I diabetes mellitus families

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Abstract

Aims/hypothesis. Type I (insulin-dependent) diabetes mellitus is the result of a T-cell regulated selective destruction of pancreatic beta cells. There is evidence that the apoptosis inducing T-cell effector, Fas ligand (FasL) could be involved in the pathogenesis of Type I diabetes, probably because FasL-mediated apoptosis is important in maintaining peripheral self-tolerance and in down-regulating an immune response. We therefore evaluated the human FasL gene *FASL* on chromosome 1q23 as a candidate susceptibility gene for Type I diabetes.

Methods. The entire *FASL* (promoter, exons 1–4 and 3'UTR) was scanned for polymorphisms using single strand conformational polymorphism-heteroduplex analysis and direct sequencing.

Results. We identified two novel polymorphisms, a *g-C843T* and a *g-A475T*, in a negative regulatory region of the promoter. A Danish Type I diabetes family collection of 1143 subjects comprising 257 families (420 affected and 252 unaffected offspring) was typed for the *g-C843T* polymorphism and for a *FASL* microsatellite. Haplotypes were established and data were analysed using the extended transmission disequilibrium test.

Conclusion/interpretation. We found no overall evidence for linkage in the presence of association of the *FASL* polymorphism to Type I diabetes and conclude that *FASL* does not contribute to the genetic susceptibility to Type I diabetes. [Diabetologia (2002) 45: 134–139]

Keywords Genetics, candidate gene, microsatellite, apoptosis, immune regulation, T cells, tolerance, SP-1, NF- κ B.

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Abbreviations: 3' UTR, 3' Untranslated region; AP-1, activation protein – 1; Egr-3, early growth response protein – 3; IFN, interferon; IRF-1, IFN regulatory factor – 1; kb, kilobases; *lpr*, lymphoproliferative; NFAT, nuclear factor of activated T cell; NF- κ B, nuclear factor - κ B; NK, natural killer; NOD, nonobese diabetic mouse; PAGE, polyacryl amide gel electrophoresis; PCR, polymerase chain reaction; SLE, systemic lupus erythematosus; SSCP, single strand conformation polymorphism; TCR, T cell receptor; TDT, transmission disequilibrium test; TNF, tumor necrosis factor; VNTR, variable number of tandem repeats

Type I (insulin-dependent) diabetes mellitus is the result of an immune mediated selective destruction of pancreatic beta cells, in which macrophages and T-helper cells play a crucial role. There is evidence in humans and in murine models of Type I diabetes that beta-cell death occurs by apoptosis [1, 2].

The apoptosis inducing T-cell effector, Fas ligand (FasL), is a 40000 M_r type II membrane protein belonging to the tumor necrosis factor (TNF) family. FasL induces apoptosis when binding to its receptor, Fas. FasL is predominantly expressed in activated T lymphocytes, in natural killer cells and in immune privileged sites, such as the testes and eye [3]. The human FasL gene, *FASL*, has four exons spanning more than 9 kb and maps to 1q23 [4]. Polymorphisms of the murine *FASL* that affect the biological activity have been described [5] as well as an 84 bp deletion in

exon 4 in one patient with systemic lupus erythematosus (SLE) and lymphoproliferative disease [6].

In murine models of Type I diabetes FasL does not seem to be an effector molecule in beta-cell apoptosis but rather plays a role in the deletion of autoreactive T cells both in the thymus and in the periphery. Hence, Fas deficient NOD *lpr/lpr* mice do not develop spontaneous diabetes and accumulate large amounts of FasL expressing abnormal double negative (Thy1 + B220 + CD4⁻ CD8⁻) T cells [7] in the lymphoid system. FasL mediated apoptosis is involved in clonal thymic deletion of thymocytes that are reactive to antigens highly expressed in the thymus [8]. The *lpr* mutation is leaky and a small amount of normal Fas is expressed in the thymus of *lpr* mice [9] and it is possible that NOD *lpr/lpr* mice do not develop diabetes because they have an intact FasL mediated deletion of autoreactive thymocytes. In humans, the dominant protective *INS* class III VNTR allele is associated with higher steady-state levels of thymic *INS* mRNA expression and could promote deletion of insulin-specific T-lymphocytes [10].

NOD *lpr/lpr* mice are also protected from adoptive transfer of diabetes, probably because host FasL-expressing double negative T cells kill the Fas-expressing transferred diabetogenic T cells. Soluble FasL induces apoptosis of potentially autoreactive CD4 + CD45RB^{low} memory T cells [11].

FAS/FASL are therefore attractive candidate susceptibility genes for Type I diabetes. We have recently scanned the entire human *FAS* for mutations and tested the frequent polymorphisms for linkage in a large Danish Type I diabetes multiplex family but we found no evidence for linkage [12]. Therefore we have scanned the human *FASL* for mutations and report our results.

Subjects and methods

Subjects. SSCP-heteroduplex analysis and direct sequencing were carried out in 20 unrelated Type I diabetes patients and 20 healthy control subjects of Danish Caucasian ancestry.

Linkage analysis was carried out in a Danish Type I diabetes family cohort (a total of 1143 individuals) comprising 154 sib pair families (a total of 725 individuals, 317 affected and 138 unaffected offspring) and 103 simplex families (a total of 418 individuals, 103 affected and 114 unaffected offspring). All index cases, defined as the first affected offspring, had onset of Type I diabetes mellitus before the age of 30 years (mean ± SD: 10.0 ± 5.5 years). All affected offspring had onset of Type I diabetes before age 30 years (mean ± SD: 10.0 ± 4.8 years). All families were unrelated. Approval for the study was obtained from the Ethics Committee of Copenhagen County.

Screening for and identification of mutations in the human *FASL*. Primers for PCR were designed from the genomic human *FASL* sequence (GenBank AC Z96050) to generate 17 overlapping PCR products 202–279 bp long (Table 1) with an average overlap of 51 bp. The PCR mixture comprised

0.8 μmol/l of each primer, 50 μmol/l dNTPs, 1.0–1.5 mmol/l MgCl₂, 0.5 U/sample Taq DNA Polymerase (Promega Corporation, Madison, Wis., USA) with 1x reaction buffer and 40 ng genomic DNA in a total reaction volume of 25 μl. The cycling conditions were 95 °C for 5 min., followed by 30 to 35 cycles of 95 °C for 30 s, 48 to 60 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min.

SSCP-heteroduplex analysis utilised the 17 PCR amplicons listed in Table 1, using the Gene Gel Excel 12.5, 24 kit and GeneGel Clean 15, 24 kit with the PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden) as described in [12]. Samples were run on the GenePhor electrophoresis unit (Amersham Pharmacia Biotech, Uppsala, Sweden) and gels were stained using the Plus One DNA Silver Staining Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) on a Hoefer Automated Gel Stainer (Pharmacia Biotech, San Francisco, Calif., USA). Samples demonstrating mobility shifts or heteroduplex formation were sequenced by cycle sequencing using the PCR product pre-sequencing kit and the thermo sequenase cycle sequencing kit (both Amersham Pharmacia Biotech, Cleveland, Ohio, USA) as described in [12].

Direct sequencing of the *FASL* promoter from position –475 to –104 (relative to the start codon) was done on an ABI Prism 310 DNA sequencer system (Perkin Elmer, Foster City, Calif., USA).

Typing for the *g-C843T* polymorphism. We typed for the *g-C843T* polymorphism by mutagenically separated PCR (MS-PCR) as described in [13] using the forward primers (mutant): 5'AATGAAAATGAAAACATTGT, (wildtype): 5'TAAAT-AAGTAAATAAATAAACTGGGCAAACCTTGAAAAT-GAAAACAACGC and reverse primer: 5'CCCACTTT-AGAAATTAGATC to yield a PCR product of 114 bp for the wildtype and 84 bp for the mutant allele. Assay conditions were: forward primers (wildtype) 1.0 μmol/l, (mutant) and reverse primer 0.5 μmol/l each, dNTPs 50 μmol/l, MgCl₂ 1.0 mmol/l, Taq DNA Polymerase (Promega Corporation) 0.5 U/sample with 1X Reaction buffer and 40 ng genomic DNA in a reaction volume of 20 μl. Cycling conditions were 95 °C for 3 min, 45 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min. PCR products were separated by gel electrophoresis on a 4% agarose gel.

***FASL* microsatellite.** Primer sequences are shown (Table 1) [14]. Using biotinylated primers we typed the *FASL* microsatellite by PCR of 40 ng genomic DNA template followed by PAGE, blotting and visualisation utilising a streptavidin-peroxidase (Sigma, St. Louis, Mo., USA) catalysed ECL reaction (Amersham Life Science, Cleveland, Ohio., USA) [12]. PCR cycling conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min. Samples homozygous for the different alleles were confirmed by sequencing on an ABI Prism 310 DNA sequencer system (Perkin Elmer, Foster City, Calif., USA).

Statistical analysis. Transmission disequilibrium test (TDT) for linkage and extended transmission disequilibrium test (ETDT) for linkage with multiallele markers were carried out [16]. The TDT is a test of association between alleles at a marker and a disease and requires linkage disequilibrium (allelic association maintained by tight linkage) between the marker alleles and the unknown gene polymorphism. The TDT detects deviations from random transmission of alleles to disease phenotypes as expected based on Hardy-Weinberg equilibrium. The TDT is still a valid test of linkage, when more than one affected offspring per family are considered. Statistical significance of 5% was chosen. Linkage disequilibri-

Table 1. Primers for human *FASL*

Primer localization	Sequence	Region ^a	Annealing temperature
75474–75753	5'ATAACAGGGGCAGCACACAGA 5'CACTGAGCCCAACTGACCAC	promoter	58°C
75660–75908	5'GATATTCTGATATTTCAAAC 5'CATAAGTCACTACACCTG		48°C
75859–76130	5'AACATAGAAAGTCCCCATCTG 5'TGCAAACCAAGTGGAAACC		52°C
76072–76290	5'AAATAAACTGGGCAAACAATG 5'GGCCAGAGAAGTCACTCC		52°C
76239–76501	5'GGGAAGAGATGATGGCAACA 5'GAGAATGGTCAGTGGGGCTAT		52°C
76475–76734	5'AGCATGCAATTATAATTCATA 5'CAAAGAAACACCCACTCA		53°C
76475–76847	5'AGCATGCAATTATAATTCATA 5'GGCAAGCTGGATCTCTCTTA	Sequencing primers	51°C
76842–77111	5'GCCTCCTCTTGAGCAGTCAGC 5'GGCGGCGGAGGTGGTAG	Promoter and exon 1	60°C
77063–77289	5'AGAAGGCCTGGTCAAAGGAG 5'GCAGGCTTACCTCTCGGAGTT	Exon 1	60°C
77243–77488	5'GGCCTGGGGATGTTT 5'GTTGGGCTCTGATTCTTTACA	Exon 1 and intron 1	54°C
77484–77762	5'ACTTTGTAAAGAATCAGAGCC 5'ATCGTCAAATAAACCAAGC	Intron 1	50°C
77732–77 934	5'GGGCTTGTTTATTGACGA 5'AGGATCATCTTTGGGAAGTCA	Intron 1 and exon 2	58°C
81961–82213	5'TGTTAGACTGTTGCCATTT 5'ACCTCTCTAGAGCCTGGTAAT	Exon 3	50°C
83326–83591	5'ATGCATGACTATTCCTTGTTG 5'CATCTTCCCCTCCATCA	Exon 4	56°C
83543–83802	5'GCCACAAGGTCTACATGAGGA 5'CATTCTCGGTGCCTGTAACAA	Exon 4 and 3'UTR	58°C
83730–83967	5'GAATCTCAGACGTTTTTCGGCT 5'CATATCTCCATTTGTCTGGCTC	3'UTR	58°C
83899–84141	5'AGGGTTCAAATGTCTGTAGCTCC 5'GAAAGACAGTCCCCCTTG-AGGT	3'UTR	58°C
84068–84294	5'GGCAGATTGAAAAGGACACCTT 5'ATCTGTTAGTTTCACCGATGGCTC	3'UTR	58°C
84252–84468	5'BiotACTTCTAAATGCATATCCTGAGCC 5'ATCTTGACCAAATGCAA CCC	Microsatellite in 3'UTR	55°C

GenBank AC Z96050

^a Including flanking exon-intron boundaries

um was calculated as the disequilibrium coefficient D [17] [$D = P_{11} - p_1q_1$], where p_1 is the frequency of the “most frequent” allele at locus 1 in the general population, q_1 is the population frequency of the “most frequent” allele at locus 2 and P_{11} is the observed frequency of the haplotype formed by these alleles.

The heterozygosity index is defined as the percentage of parents who are heterozygous for the polymorphism that is being tested.

Results

Mutation detection by SSCP-heteroduplex analysis and direct sequencing. The entire human *FASL* (promoter, exons 1–4 including exon-intron boundaries and the 3'UTR) was screened for mutations by SSCP-heteroduplex analysis followed by direct sequencing of polymorphic variants in 40 unrelated Danish Caucasians (20 Type I diabetic patients and 20 healthy control subjects), using the 17 PCR amplicons established from the genomic *FASL* sequence and listed in Table 1. The *FASL* promoter was directly sequenced from position –475 to –104 (relative to the start codon) in the same 40 subjects. In the entire human *FASL* we identified a total of two novel polymorphisms, a *g-C843T* and a *g-A475T*, both situated in the promoter.

Linkage analysis

***FASL* promoter polymorphisms.** The TDT [15] was applied to analyse the *g-C843T* polymorphism for linkage in the presence of association to Type I diabetes. The parental heterozygosity index was 0.51. We found no evidence for linkage, $P_{TDT} \geq 0.13$ (Table 2).

A high throughput typing method for the *g-A475T* polymorphism could not be established. To type for this polymorphism, which does not lead to an altered restriction enzyme site, we have tried to establish an MS-PCR assay and a single nucleotide primer extension analysis. We could not type for the polymorphism by SSCP, as the SSCP pattern was not unequivocal. The frequencies of the A and T allele were 0.82 and 0.18, respectively in our screening panel of 20 Type I diabetic patients and 20 control subjects. The T allele was in linkage disequilibrium with the T allele of the *g-C843T* polymorphism.

***FASL* microsatellite.** The Danish family collection was typed for a *FASL* microsatellite (GT repeat polymorphism) [14] localized in the 3'UTR within 600 bp of exon 4. Seven alleles ranging from 14 to 22 GT repeats were observed. The 16 and 17 repeat alleles were the most frequent with allelic frequencies of 0.795 and 0.18, respectively. The parental heterozy-

Table 2. *FASL* promoter *g-C843T* polymorphism: allelic transmission patterns from heterozygous parents to offspring

	C	T	χ^2
Affected	160	134	2.3
Index cases	103	87	1.4
Unaffected	99	89	0.5

Allele frequencies in the parental generation were 0.6 and 0.4 for the C and T allele, respectively and parental heterozygosity index was 0.51. (TDT) $p \geq 0.13, \geq 0.25, \geq 0.47$, for affected, index cases and unaffected, respectively

gosity index was 0.32. Data were analysed for linkage in the presence of association using the ETDT [16] (Table 3; $P_{\text{ETDT}} \geq 0.015$). The results showed preferential transmission of the 16 allele from heterozygous parents to all offspring (58% and 54% to affected and unaffected offspring, respectively) and showed no significant difference in transmission patterns between affected and unaffected offspring when compared by the χ^2 test ($p \geq 0.5$).

FASL haplotypes. Haplotypes were established from the *g-C843T* polymorphism and the 16 and 17 alleles of the *FASL* microsatellite and tested for linkage to Type I diabetes using the ETDT (Table 4). The parental heterozygosity index was 0.63, and we found no evidence for linkage, $P_{\text{ETDT}} \geq 0.2$.

Linkage disequilibrium

The disequilibrium coefficient D [17] for the *g-C843T* polymorphism and the *FASL* microsatellite was calculated to be 0.05.

Discussion

We have screened the entire human *FASL* for mutations by SSCP-heteroduplex analysis followed by direct sequencing of polymorphic variants.

In addition we have sequenced the promoter from -475 to -104, an area containing the NFAT binding site essential for *FASL* transcription in T-cell activa-

tion via the TCR, CD3 and CD4 [18] as well as binding sites for nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$), early growth response protein - 3 (Egr-3), IFN regulatory factor - 1 (IRF-1) and activating protein-1 (AP-1)[3].

We identified two novel polymorphisms in the promoter, a *g-C843T* and a *g-A475T*. These are located in a negative regulatory region of the promoter [18] but do not alter transcription factor bindings sites.

A mutagenically separated PCR (MS-PCR) assay [13] was established to type a large Danish Type I diabetic multiplex family collection for the informative *g-C843T* polymorphism and data were analysed for linkage in the presence of association to Type I diabetes by the transmission disequilibrium test [15]. However, we did not find evidence for linkage.

We also typed the Danish family collection for a *FASL* microsatellite [14] localized in the 3'UTR of *FASL*, and analysed data for linkage to Type I diabetes using the extended transmission disequilibrium test [16]. However, these results do not indicate linkage in the presence of association as one could be led to believe if testing transmissions only to affected offspring. When considering transmissions from heterozygous parents to all offspring, the results showed preferential transmission of the most frequent allele, 16 repeat allele, to affected and as well as unaffected offspring and no significant difference in transmission patterns of the 16 repeat allele. The skewed transmission of the 16 allele could be due to meiotic segregation distortion and not to linkage to Type I diabetes. This emphasizes the importance in testing the transmissions to unaffected as well as affected offspring [15].

We then established haplotypes from the *g-C843T* polymorphism and the 16 and 17 alleles of the *FASL* microsatellite and tested these for linkage using the ETDT. The haplotypes were highly informative, since 63% of the parents were heterozygous. We found no evidence for linkage of the *FASL* haplotypes to Type I diabetes.

The disequilibrium coefficient D [17] for the *g-C843T* polymorphism and the *FASL* microsatellite was calculated to be 0.05, which indicates only weak linkage disequilibrium between these two polymorphisms situated less than 9 kb apart.

Table 3. *FASL* microsatellite; allelic transmission patterns of the two most frequent alleles from heterozygous parents to offspring

	Allele size (repeat units)	Transmitted	Non transmitted	χ^2	p value ^a
Affected	16	117	85	5.07	0.02
	17	78	102	3.2	0.07
Index cases	16	71	54	2.31	0.13
	17	51	59	0.58	0.45
Unaffected	16	61	51	0.89	0.34
	17	42	58	2.56	0.11

Parental heterozygosity index 0.32

^a Uncorrected p values. (ETDT) $p \geq 0.015, \geq 0.13, \geq 0.24$ for affected, index cases and unaffected, respectively

Table 4. *FASL* haplotype (established from the *g-C843T* promoter polymorphism and the two most frequent alleles of the microsatellite in the 3' UTR of *FASL*) transmissions

	Haplotype	Transmitted	Non transmitted	χ^2	<i>p</i> value ^a
Affected	T16	112	124	0.6	0.43
	T17	36	44	0.8	0.37
	C16	150	118	3.8	0.05
	C17	42	54	1.5	0.22
Index cases	T16	74	78	0.1	0.75
	T17	22	27	0.5	0.48
	C16	89	79	0.6	0.44
	C17	30	31	0.02	0.90
Unaffected	T16	76	76	0	1.0
	T17	12	19	1.58	0.21
	C16	87	80	0.29	0.59
	C17	27	27	0	1.0

Transmission patterns of haplotypes from heterozygous parents to offspring. The frequencies of haplotypes in the parental

generation were: T16: 0.34, T17: 0.07, C16: 0.50, C17: 0.09 and the parental heterozygosity index was 0.63

^a Uncorrected *p* values. (ETDT) *p* \geq 0.2

Power estimates assuming a 12.5% deviation show that the probability of making a type II error at 5% in the statistical analysis is low due to the many observed transmissions.

The risk that we have overlooked polymorphisms in the human *FASL* that are linked to Type I diabetes is small, since the sensitivity and specificity of the SSCP-heteroduplex analysis in our laboratory is high [12] and in addition we have sequenced the 3' area of the promoter involved in *FASL* expression in T cells in 40 subjects. Minor susceptibility genes are expected to modify risk and are likely to be frequent in the general population. We have identified another polymorphism, *g-A475T*, which was checked by sequencing in the screening panel only. This polymorphism is in linkage disequilibrium with the *g-C843T* polymorphism and we therefore find it unlikely that the *g-A475T* polymorphism should independently be associated with Type I diabetes.

In summary, we have scanned the entire human *FASL* and identified two novel polymorphisms in a negative regulatory region of the *FASL* promoter. These are, however, not linked under the assumption of association with Type I diabetes but could be relevant for the understanding of the genetic susceptibility to other immune mediated disorders.

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