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CLINICAL STUDY

No association of two Fas gene polymorphisms with Hashimoto's thyroiditis and Graves' disease

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Abstract

Background: Apoptosis is a joint pathogenic process underlying autoimmune thyroid disease. Increased programmed cell death in thyrocytes causes hypothyroidism in Hashimoto's thyroiditis, whereas in Graves' disease infiltrating lymphocytes undergo apoptosis while thyrocytes appear to proliferate under protection of anti-apoptotic signals. The Fas/Fas ligand cascade represents a major pathway initiating apoptosis. Its role in autoimmunity is well studied and genetic polymorphisms in gene loci of Fas and its ligand have been shown to be associated with autoimmune diseases.
Objective: Due to the functional relevance of the Fas pathway in autoimmune thyroid disease we were interested in the possible contribution of polymorphisms in the Fas gene to the genetic risk of thyroid autoimmunity, which so far is mainly, but incompletely, attributed to the HLA DQ region and polymorphisms in the CTLA-4 gene.
Design: We genotyped Caucasian families with at least one offspring affected by Hashimoto's thyroiditis ($n = 95$) and Graves' disease ($n = 109$) for two Fas gene polymorphisms (g-670 G → A in the promoter region, g-154 C → T in exon 7).
Methods: Extended transmission disequilibrium and χ^2 testing were performed.
Results: Neither polymorphism alone ($P = 0.44$ and $P = 0.70$) nor the promoter/exon 7 haplotypes ($P = 0.86$) were associated with Hashimoto's thyroiditis. No association with Graves' disease was observed for the promoter polymorphism ($P = 0.91$) and exon 7 ($P = 0.65$) or the promoter/exon 7 haplotypes ($P = 0.80$).
Conclusion: In summary, our data do not suggest any significant contribution of common genetic Fas variants to the genetic risk of developing Hashimoto's thyroiditis or Graves' disease.

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Introduction

The two most common autoimmune diseases are the ones affecting the thyroid: Graves' disease (GD) and Hashimoto's thyroiditis (HT). HT is characterized by diffuse enlargement of the thyroid gland, lymphocytic infiltration creating a cytokine milieu of Th1 mediators and thyroid dysfunction that in its extent strictly correlates with the expression of pro-apoptotic molecules (1). In contrast, GD is caused by autoantibodies to thyroid-stimulating hormone (TSH) receptor, histologically presenting increased apoptosis of infiltrating lymphocytes and the survival of thyrocytes, resulting in hyperthyroidism and goiter.

In organ-specific autoimmunity, apoptosis causes tissue destruction by altering target organ susceptibility and activating T cells. Among various apoptosis-inducing signals in thyroid autoimmunity the Fas/Fas ligand (FasL) pathway appears to be most relevant. Thyrocytes in both autoimmune thyroid diseases (AITDs) express Fas and are susceptible to FasL-induced apoptosis (2), but the balance of pro- and anti-apoptotic signals differs between HT and GD.

Apoptotic thyrocytes in HT show increased expression of both Fas and FasL together with down-regulation of anti-apoptotic Bcl-2, promoting mutual induction of apoptosis within the follicular epithelia (3, 4). In contrast, thyrocytes of GD glands show less Fas/FasL and more Bcl-2 than HT thyrocytes. But infiltrating T lymphocytes (ITLs) express increased levels of Fas/FasL and decreased Bcl-2 levels, thereby favoring thyrocyte survival and apoptosis of ITLs (3, 4).

Therefore Fas/FasL-mediated apopotosis may occur in both immune and thyroid cells, whereby the different degree of cell death determines the opposite phenotypic outcome of HT and GD (5).

Fas is a type I transmembrane protein that belongs to the tumor necrosis factor family. Its gene maps to chromosome 10q24.1 and consists of nine exons spanning about 26 kb (6). It is expressed on the surface of many types of cells, such as lymphocytes, epithelial cells and normal thyrocytes but to a significantly elevated level in autoimmune thyroids (4).

A molecular scan of the entire Fas gene in a Danish population detected 15 mutations (7). Point mutations were found in children affected by lymphoproliferative disorders (8) and somatic frameshift mutations were present in the mantle zone and germinal centers of HT glands (9).

Both thyroid autoimmune diseases are multifactorial and of genetic origin (10, 11). But despite numerous studies and the demonstration of the importance of the HLA DQ region on chromosome 6 (12) and polymorphisms of the CTLA-4 gene on chromosome 2q33 (13), the exact nature of the genetic susceptibility is still unresolved.

Using linkage disequilibrium mapping, some polymorphisms and mutations in the Fas gene were shown to be associated with Sjögren's disease (14) and neoplastic disorders, e.g. lung and gastric carcinoma. Furthermore, several polymorphisms within the Fas promoter are suggested to be associated with multiple sclerosis (MS) (15, 16), rheumatoid arthritis and systemic lupus erythematosus (SLE) (17–19).

One of the well-described polymorphisms has not only been shown to be associated with SLE and MS but is also of functional significance: g-670 G → A in the promoter region (20). The A allele abolishes the binding site for the nuclear transcription element gamma activation site that is important in interferon signaling.

Based on the functional relevance of Fas in the pathogenesis of AITDs we examined this single-nucleotide polymorphism and a second variant (g-154 C → T in exon 7) to obtain haplotypes as markers for a potential association with HT and GD in an extended family study.

Patients and methods

We studied 209 families with thyroid autoimmunity comprising a total of 730 individuals of Caucasian origin. In $n = 86$ ($n = 14$) families one (two) offspring were affected by HT. In $n = 105$ ($n = 4$) families one (two) offspring had GD. Ninety-eight GD and seven HT families were recruited at the Endocrine Outpatient Clinic of the University Hospital, Frankfurt am Main (Germany), 93 HT and 11 GD families at the Department of Pediatrics, University La Sapienza, Rome (Italy).

HT was defined on the basis of the presence of anti-thyroid peroxidase antibodies and/or anti- thyroglobulin autoantibodies, and thyroid ultrasound with reduced echogenicity compatible with thyroiditis, regardless of the thyroid function. GD was defined by clinical and biochemical hyperthyroidism and positivity for anti-TSH receptor antibodies.

The male:female ratio was 1:5 among HT and 1:4.5 among GD patients In $n = 22$ ($n = 8$) families one parent was also affected by HT (GD).

Genomic DNA was prepared from 10 ml whole venous blood either by salt extraction according to standard protocols or with a QIAamp blood kit (Qiagen GmbH, Hilden, Germany). DNA was amplified using PCR in a total volume of 25 μl containing 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris–HCl, 8 mmol/l dNTPs, 25 pmol each primer, 200 ng genomic DNA and 1.25 U Taq polymerase (Promega, Madison, WI, USA). Standard PCR conditions were as follows: initial denaturation for 3 min at 94 °C, 30 cycles of 94 °C, annealing temperature (see below) and 72 °C for each 1 min, and final extension for 10 min at 72 °C.

Primers were designed according to the published sequence (Genbank accession number AC X82279-X82286). A 474 bp fragment containing the promoter polymorphism g-670 was amplified using the primers 5′-CCAAAGGAATACTGAAACC-3′ and 5′-CACTCAGAGAAAGACTTGC-3′ (annealing temperature 55 °C). Five microliters of the PCR product were incubated with 5 U BstNI (New England Biolabs, Beverly, MA, USA) in a total volume of 20 μl for 3 h at 60 °C. One restriction site generated a 210 bp fragment in every probe, indicating successful digestion. The remaining 264 bp were also cut to 184 bp in the case of the G allele and remained undigested in the case of the A allele. In heterozygous subjects three bands were visible: 264 (A), 210 and 184 (G) bp.

A 178 bp fragment containing the g-154 exon polymorphism was amplified using the primers 5′-TCTCACATGCATTCTACAAGG-3′ and 5′-TTTCAAGGAAAGCTGATACC-3′ (annealing temperature 56 °C). Digestion with DraI (5 U for 3 h at 37 °C) resulted in two fragments of 128 and 50 bp for the T allele. In samples heterozygous for the respective restriction sites, both digested and undigested DNA fragments were visible. Amplified DNA fragments and digestion products were separated on 2.5% agarose gels and visualized by ethidium bromide.

Transmission disequilibrium testing (TDT) was performed to detect preferential transmission of Fas alleles to affected offspring (21). To assess the potential role of extended Fas promoter/exon 7 haplotypes, we performed indirect haplotyping using genotype information from all available members of the respective family followed by extended TDT (ETDT) (22). Extended haplotypes could not be unequivocally ascertained in 19 HT and 14 GD families and these families had to be excluded from ETDT. χ^2 testing was performed to compare the subsets of families stratified for HLA DQ2 haplotypes and parental origin of alleles as well.

Results

No significant transmission disequilibrium was observed in families with HT for the promoter ($P_{TDT} = 0.44$) or the exon 7 ($P_{TDT} = 0.70$) polymorphism (Table 1) or the extended promoter/exon 7 ($P_{ETDT} = 0.86$) haplotypes. Considering also those HT families with uncertain haplotypes, we did not detect any transmission disequilibrium either (Table 2). In families with GD no transmission disequilibrium was observed for the promoter ($P_{TDT} = 0.91$), the exon 7 polymorphism ($P_{TDT} = 0.65$) (Table 1) or the extended promoter/exon 7 haplotypes ($P_{ETDT} = 0.80$) (Table 2). We also did not observe any difference between Italian and German families (data not shown).

Discussion

The data obtained in this first study of Fas gene polymorphisms in families affected by GD and HT demonstrate no evidence of an association as investigated by TDT and ETDT. Neither polymorphism alone nor the extended promoter/exon 7 haplotypes showed any difference from expected values due to transmission by chance. In conclusion, our data do not suggest any significant contribution of the two genetic variants in the Fas gene to the genetic risk of developing thyroid autoimmune disease.

Table 1 Transmission of Fas polymorphisms from heterozygous parents to offspring with HT and GD.

Disease	Polymorphism	Allele	Transmitted (%)	Not transmitted (%)	P_{TDT}
HT	Promoter G670A	A	45 (54)	38 (46)	0.44
		G	38 (46)	45 (54)	
	Exon 7 C154T	C	32 (50)	32 (50)	0.70
		T	39 (50)	32 (50)	
GD	Promoter G670A	A	38 (49)	39 (51)	0.91
		G	39 (51)	38 (49)	
	Exon 7 C154T	C	40 (53)	36 (47)	0.65
		T	36 (47)	40 (53)	

Table 2 Transmission analysis of extended Fas promoter/exon 7 haplotypes in families with HT and GD.

Disease	Promoter–exon 7 haplotype	Transmitted (%)	Not transmitted (%)	P_{TDT}	P_{ETDT}
HT	A–C	27 (46)	32 (54)	0.52	0.86
	A–T	12 (57)	9 (43)	0.52	
	G–C	22 (54)	19 (46)	0.64	
	G–T	22 (48)	23 (52)	0.88	
GD	A–C	27 (52)	25 (48)	0.78	0.80
	A–T	10 (36)	18 (64)	0.13	
	G–C	20 (54)	17 (46)	0.62	
	G–T	27 (53)	24 (47)	0.67	

A previous study on Fas polymorphisms did not show association with type 1 diabetes either. But significant linkage disequilibrium is still suggested for neoplastic disorders and other autoimmune diseases. Additionally, somatic frameshift mutations of Fas are present in the mantle zone and germinal centers of HT glands (9).

Even though neither the investigated Fas polymorphisms nor the previously described family study on the FasL gene (23) showed significant involvement in genetic susceptibility to AITD, the pathogenic relevance of their gene products remains undisputed.

Functional data strongly support a role for Fas/FasL in the development of HT and GD. As shown in studies of the cytokine milieu, cell membrane morphology and the effects of *in vitro* cytokine stimulation the opposite clinical and cytological presentation of HT and GD result from apoptosis induction via Fas/FasL (4). Whereas increased activity of Fas/FasL strictly correlates with the extent of thyrocyte destruction in HT, ITLs undergo excessive Fas/FasL apoptosis while thyrocytes proliferate under protection of anti-apoptotic molecules (24) in GD.

Considering its pivotal role, any variants of the Fas/FasL system may be involved in the pathogenesis of thyroid autoimmunity and should be investigated at the genomic as well as proteomic level.

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