

A Genomic Point Mutation in the Extracellular Domain of the Thyrotropin Receptor in Patients with Graves' Ophthalmopathy*

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ABSTRACT

Orbital and pretibial fibroblasts are targets of autoimmune attack in Graves' ophthalmopathy (GO) and pretibial dermopathy (PTD). The fibroblast autoantigen involved in these peripheral manifestations of Graves' disease and the reason for the association of GO and PTD with hyperthyroidism are unknown. RNA encoding the full-length extracellular domain of the TSH receptor has been demonstrated in orbital and dermal fibroblasts from patients with GO and normal subjects, suggesting a possible antigenic link between fibroblasts and thyrocytes.

RNA was isolated from cultured orbital, pretibial, and abdominal fibroblasts obtained from patients with severe GO (n = 22) and normal subjects (n = 5). RNA was reverse transcribed, and the resulting cDNA was amplified by the polymerase chain reaction, using primers spanning overlapping regions of the entire extracellular domain of the TSH

receptor. Nucleotide sequence analysis showed an A for C substitution in the first position of codon 52 in 2 of the patients, both of whom had GO, PTD, and acropachy. Genomic DNA isolated from the 2 affected patients, and not from an additional 12 normal subjects, revealed the codon 52 mutation by direct sequencing and *AclI* restriction enzyme digestions.

In conclusion, we have demonstrated the presence of a genomic point mutation, leading to a threonine for proline amino acid shift in the predicted peptide, in the extracellular domain of the TSH receptor in two patients with severe GO, PTD, acropachy, and high thyroid-stimulating immunoglobulin levels. RNA encoding this mutant product was demonstrated in the fibroblasts of these patients. We suggest that the TSH receptor may be an important fibroblast autoantigen in GO and PTD, and that this mutant form of the receptor may have unique immunogenic properties. (*J Clin Endocrinol Metab* 78: 256-260, 1994)

ORBITAL and pretibial fibroblasts are targets of autoimmune attack in Graves' ophthalmopathy (GO) and pretibial dermopathy (PTD) (1). However, the association of the hyperthyroidism of Graves' disease with these connective tissue-based extrathyroidal manifestations has long been enigmatic. We have shown previously that fibroblasts contain RNA encoding the extracellular domain of the TSH receptor (2). Similar findings (in retroorbital tissue samples) have been reported by another group (3). If translated into protein, this fibroblast autoantigen might be recognized by lymphocytes directed against the TSH receptor in Graves' disease. The subsequent infiltration of the orbit and skin by activated cytokine-producing lymphocytes would probably stimulate glycosaminoglycan synthesis and proliferation of the resident fibroblasts (1). The resulting histological changes would be those characteristic of the involved tissues in GO and PTD (4).

Autoantigens are expected to be normal in autoimmune diseases; the abnormality is most likely to be found in the immune system itself. However, a somatic point mutation in the extracellular domain of the TSH receptor has been re-

ported in thyroid RNA of a patient with Graves' disease (5). As the somatic mutation is located in a putative ligand-binding domain, the researchers speculated that the mutation has a causal relationship to the patient's thyroid disorder. We wished to determine whether there might be a mutation in the TSH receptor gene in fibroblasts of patients with GO. In this study we demonstrate a genomic point mutation in the extracellular domain of the TSH receptor in two patients with GO, PTD, acropachy, and high thyroid-stimulating immunoglobulin (TSI) levels. RNA encoding the product of this mutant gene is present in fibroblasts from these patients. Because of the high levels of antibody activity directed against the TSH receptor in these two patients and the severity of their connective tissue manifestations, we hypothesize that the protein product of their mutant TSH receptor gene may have unique immunogenic properties.

Materials and Methods

Subjects

Orbital connective tissue was obtained from patients with severe GO (n = 22) in the course of transantral orbital decompression surgery (see Table 1 for clinical details). Normal orbital connective tissue was obtained from individuals with no history of thyroid disease who underwent orbital surgery for unrelated conditions (n = 5). Pretibial (n = 2) and abdominal (n = 1) skin biopsies were obtained by written consent and Institutional Review Board approval from two of the patients with GO who also had PTD (patients 1 and 18; Table 1). In addition, a fine

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TABLE 1. Clinical characteristics and TSH receptor analysis in 22 patients with severe GO

Patient no.	Age (yr)	Sex	Presence of PTD	Presence of acropachy	TSI index ^a	Presence of codon 52 mutation
1	39	F	+	+	16	+
2	67	F	-	-	ND ^b	-
3	34	M	-	-	8.9	-
4	34	F	+	-	1.9	-
5	58	F	+	+	ND	-
6	50	F	+	+	22	+
7	65	F	-	-	ND	-
8	55	F	+	-	19	-
9	85	F	-	-	ND	-
10	55	M	-	-	8.4	-
11	45	F	-	-	13	-
12	68	M	-	-	17	-
13	64	M	-	-	9.4	-
14	62	F	-	-	8.7	-
15	45	F	-	-	18	-
16	63	F	-	-	14	-
17	53	F	-	-	8.7	-
18	55	M	+	-	17	-
19	51	F	-	-	8.8	-
20	54	F	-	-	5.7	-
21	61	M	-	-	6.4	-
22	50	F	+	+	15	-

^a TSI index at time of orbital decompression surgery (normal = 0.0-1.3).

^b Not done.

needle aspiration biopsy of the thyroid was obtained from patient 1. Peripheral blood samples were collected from additional patients with severe GO (n = 3) and normal subjects (n = 12). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using standard procedures (6). Serum TSI activity was determined using a standard assay that measures cAMP generation in cultured FRTL-5 thyroid cells (7).

Cell culture

Fibroblast cultures were established from biopsy specimens, as described previously (8). Briefly, tissue explants were minced and placed directly on plastic culture dishes, and fibroblasts were allowed to proliferate. Fibroblast cultures were propagated in medium 199 containing 20% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin (100 IU/mL), and gentamycin (20 µg/mL) in a humidified 5% CO₂ incubator at 37 C. All cell cultures established in this fashion stain positively for a specific antifibroblast antigen (Dakopatts Corp., Santa Barbara, CA). Cultures were maintained in 75-mm² flasks with medium 199 containing 10% fetal bovine serum and were used between the first and fifth passages. Cultured Chinese hamster ovary (CHO) cells, transfected with either plasmid containing the human TSH receptor (JPO9 line) or the untransfected counterpart (JPO₂ line) (9), and Fisher rat thyroid cells (FRTL-5, CRL 8305, American Type Culture Collection, Rockville, MD) were used as control cell lines.

RNA/DNA amplification with transcript sequencing

Total RNA was isolated directly from 1 × 10⁶ fibroblast cells using guanidinium thiocyanate and cesium chloride (10). First strand cDNA synthesis was performed using 20 µg heat-denatured total RNA and random hexamers (11). The resulting cDNA was amplified by the polymerase chain reaction (PCR) with primers spanning overlapping regions of the entire extracellular domain of the TSH receptor. Oligonucleotide primers 2 and 3 were used for amplification of the cDNA fragment containing the base pair (bp) substitution (Table 2). PCR amplification was performed with 4 µL cDNA template and 5.0 pmol of each PCR primer in 10 mmol Tris-HCl (pH 8.3), 50 mmol KCl, 1.5 mmol MgCl₂, 200 µmol/L of each deoxy-NTP, and 1 U Ampli-Taq DNA

TABLE 2. Oligonucleotide sequences and descriptions of primers

Primer no.	Oligonucleotide sequence	Description ^a
1	5'-TAATACGACTCACTATAG-GGAGATCCCGTGGAAAAT-GAGGCC-3'	Residues 90-108: sense strand ^b
2	5'-ATTTAGGTGACACTATAG-AATACTCCCGTGGAAAAT-GAGGCC-3'	Residues -90-108: sense strand ^c
3	5'-TAATACGACTCACTATAG-GGAGACAGGTGTTTCTTG-CTATCAG-3'	Residues 854-873: antisense strand
4	5'-TAATACGACTCACTATAG-GGAGAAAATGCATGACTT-GGAAT-3'	Residues 299-316: antisense strand ^b
5	5'-CATACACATACGATTTAG-GTGACACTATAGAATACA-GAGTCTGCGTACTGG-3'	Residues 255-270: antisense strand ^c

^a Nucleotide positions are according to Nagayama *et al.* (12).

^b T7 phage promoter as first 23 nucleotides.

^c Sp6 phage promoter as first 23 nucleotides.

^d Sp6 phage promoter as first 35 nucleotides.

polymerase (Perkin-Elmer/Cetus, Norwalk, CT) in a final volume of 50 µL. The amplification reaction for cDNA was 35 cycles (Perkin-Elmer thermal cycler 801-0150); each cycle consisted of 94 C for 1 min (denaturation), 55 C for 2 min (annealing), and 72 C for 3 min (elongation), followed by a final 10-min extension at 72 C.

Genomic DNA was isolated and purified from 1 × 10⁵ fibroblasts or PBMC from 7 mL whole blood by standard procedures (6). In addition, genomic DNA was isolated from cells obtained by a thyroid fine needle aspiration and digested with proteinase-K (12). The procedure for amplification of genomic DNA was identical to that described above, except that 1 µL DNA and PCR primers 1 and 5 (Table 2; 2.5 pmol each) were amplified using 0.5 U Ampli-Taq polymerase in a total volume of 20 µL; the annealing temperature was 55 C. For the thyroid fine needle aspiration sample, 20 µL cell lysate were amplified with PCR primers 2 and 4 (50 pmol each) and 2 U Ampli-Taq DNA polymerase in a total volume of 80 µL; 4 µL of this product were reamplified with primers 1 and 5 (10 pmol each) and 1 U Ampli-Taq DNA polymerase in a total volume of 50 µL. Amplification reaction conditions were the same as described above.

For direct sequencing of amplified segments from cDNA or genomic DNA, 3 µL of the amplified material were added to 17 µL transcription mixture [40 mmol/L Tris-HCl (pH 7.5), 6 mmol/L MgCl₂, 2 mmol/L spermidine, 10 mmol/L NaCl, 0.5 mmol/L of each ribonucleoside triphosphate, 1.6 U/mL RNasin, 10 mmol/L dithiothreitol (DTT), 10 U T7 RNA polymerase, and diethylpyrocarbonate-treated H₂O] (13). Samples were incubated for 1 h at 37 C, and the reaction was stopped by heating at 65 C for 10 min. A 2-µL aliquot of the transcription reaction was added to 10 µL annealing buffer (250 mmol/L KCl and 10 mmol/L Tris-HCl, pH 8.3) containing a ³²P end-labeled reverse transcription primer. Samples were heated at 80 C for 3 min and then annealed for 45 min at 45 C. A 2-µL aliquot of the primer RNA template solution was added to 3.3 µL reverse transcriptase buffer [24 mmol/L Tris-HCl (pH 8.3), 16 mmol/L MgCl₂, 8 mmol/L DTT, 0.4 mmol/L of each deoxyribonucleoside triphosphate, and 0.4 mmol/L thymidine triphosphate] containing 5 U avian myeloma virus reverse transcriptase and 1 µL of a dideoxyribonucleoside triphosphate. The sample was incubated at 50 C for 45 min, and the reaction was stopped by adding 2.5 µL formamide (100%) with bromophenol blue (0.3%) and xylene cyanol FF. Samples were boiled for 3 min, and a 1.5-µL aliquot was loaded onto a 100-cm sequencing gel and separated by electrophoresis (~15,000 V-h). Autoradiography was performed, and gels were read manually.

Restriction enzyme digestions

Each cDNA amplification product (20 μ L) was digested at 37 C overnight in 1 \times NEB buffer 3 (100 mmol NaCl, 50 mmol Tris-HCl, 10 mmol MgCl₂, and 1 mmol DTT, pH 7.9, at 25 C) with 15 U *Ac*I enzyme (551S, New England Biolabs, Inc., Beverly, MA) in a total reaction volume of 30 μ L. Digestion products (15 μ L) were resolved on 2% agarose gels (wild-type cDNA produces fragments of 44, 49, 92, and 642 bp; mutant cDNA produces fragments of 734, 44, and 49 bp; undigested cDNA fragment is 827 bp). The digestion of the amplified product from genomic DNA was identical, except that each amplification product (10 μ L) was digested with 10 U *Ac*I enzyme in a total reaction volume of 20 μ L that was entirely loaded onto a 4% agarose gel (fragments from wild-type DNA are 44, 49, 55, and 92 bp; mutant DNA produces fragments of 146, 44, and 49 bp; undigested DNA fragment is 240 bp).

Results

For the PCR-based amplification of TSH receptor RNA, primers were designed to amplify overlapping fragments of cDNA encompassing the full-length extracellular domain of the TSH receptor. The entire 1183-bp extracellular domain was sequenced using RNA derived from cultured retroocular fibroblasts obtained from 22 patients with GO and 5 normal subjects and from cultured pretibial (n = 2) and abdominal (n = 1) skin fibroblasts from 2 of the patients with GO who also had PTD (patients 1 and 18; Table 1). In each sample, we found nucleotide sequence corresponding to the full-length extracellular domain reported by Nagayama *et al.* (14), except for an A for C substitution in the first position of codon 52 that was present in fibroblast RNA samples from 2 of the patients studied (patients 1 and 6; Fig. 1). The wild-type receptor sequence (CCC at codon 52) was also present in each of these 2 samples. The reverse transcription step and cDNA amplification were repeated several times with identical results in both of these patients. The presence of the mutation was subsequently verified in RNA from both patients using *Ac*I restriction enzyme digestions of amplified cDNA (Fig. 2). This mutation leads to a threonine for proline amino acid shift in the predicted peptide. Proline, the wild-type amino acid at codon 52, is highly conserved; its presence in this position has been demonstrated in both the rat (15) and dog (16) TSH receptor sequences.

To determine whether the mutation in codon 52 of the TSH receptor is a germline mutation, we examined genomic DNA from patients 1 and 6 (Table 1). *Ac*I restriction enzyme digestions of amplified genomic DNA from patient 1 demonstrated both the wild-type and the mutant nucleotide sequences at the first position of codon 52 in cultured retroocular, pretibial skin, and abdominal skin fibroblasts; cells

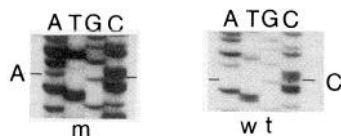


FIG. 1. Sequence analysis of the two forms of TSH receptor. Amplified PCR fragments from cDNA template were sequenced as described in *Materials and Methods*. The wild-type (wt) form has a cystine at the first position of codon 52 (noted by markers; right). The mutant (m) form (cDNA template was derived from patient 1) has both a cystine and an adenine at this position (left).

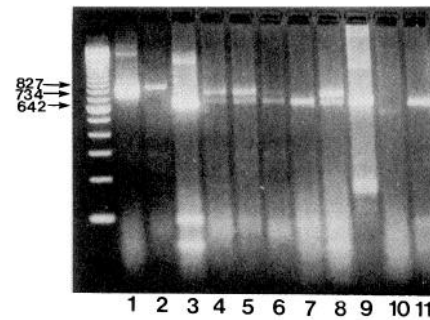


FIG. 2. *Ac*I restriction enzyme digestions of PCR fragments amplified from cDNA templates. The undigested fragment size is 827 bp. *Ac*I digestion of wild-type sequence produces 642-, 92-, 49-, and 44-bp fragments; the mutant allele produces an additional 734-bp fragment. Derivation of cDNA templates in lanes following the 100-bp ladder is as follows: 1) JP09 (TSH receptor-positive CHO cells; undigested), 2) patient 1 (undigested), 3) JP09, 4) patient 1 orbital fibroblasts, 5) patient 1 pretibial skin fibroblasts, 6) patient 1 abdominal skin fibroblasts, 7) orbital fibroblasts from a normal subject, 8) patient 6 orbital fibroblasts, 9) rat thyroid cells (FRTL-5), 10) JP02 (TSH receptor-negative CHO cells), and 11) patient 8 orbital fibroblasts.

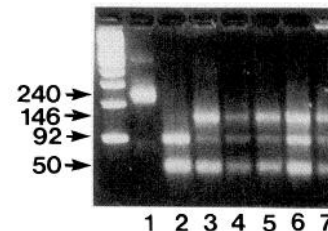


FIG. 3. *Ac*I restriction enzyme digestions of PCR fragments amplified from genomic DNA template from patient 1. The undigested fragment is 240 bp. *Ac*I digestion of wild-type sequence produces fragments of 44, 50, 54, and 92 bp; the mutant allele produces an additional 146-bp band. Derivation of genomic DNA templates in lanes following the 100-bp ladder are as follows: 1) patient 1 orbital fibroblasts (undigested), 2) orbital fibroblasts from a normal subject, 3) patient 1 orbital fibroblasts, 4) patient 1 pretibial fibroblasts, 5) patient 1 abdominal fibroblasts, 6) patient 1 thyroid fine needle aspiration sample, and 7) patient 1 PBMC.

from a thyroid fine needle aspiration sample; and PBMC (Fig. 3). The reproducible findings of the mutation in cells from several sites obtained from the same patient and its consistent heterozygosity, mitigate strongly against a *Taq* polymerase error being responsible for these results.

The same restriction enzyme digestion strategy was used to examine genomic DNA from PBMC of 12 normal subjects. None was found to have the mutation at codon 52. These 12 normal subjects, in addition to the 5 normal subjects whose fibroblast RNA was entirely sequenced in the area of codon 52, bring to a total of 17 the normal subjects studied; none had the mutation.

In addition to the mutation in the first position of codon 52, we observed a polymorphism at nucleotide position 661 (third position of codon 187). We found 13 of 22 patients with GO to be homozygous for thymidine at this position, 1 GO patient was homozygous for cytosine, and the remaining GO patients were heterozygous (thymidine/cytosine) at 661. Four of the normal subjects were homozygous for thymidine at nucleotide position 661; the remaining normal subject was heterozygous (thymidine/cytosine). We consider this varia-

tion, found in both normal individuals and GO patients, to be a polymorphism because it would not result in a change in the wild-type amino acid (asparagine) at codon 187.

Discussion

We report the presence of a germline mutation in the first position of codon 52 of the TSH receptor in two patients with severe GO, PTD, acropachy, and high TSI levels. Several groups of investigators have identified regions on the N-terminal region of the extracellular domain of the TSH receptor that appear to have epitopes for binding of TSH or antibodies directed against this receptor. Amino acid residues 30–33, 34–37, 42–45, 52–56, and 58–61 (17); 32–56 (18); and 38–45 (19) have been specifically implicated as important stimulatory antibody-binding regions. However, it is likely that these epitopes are conformational and include multiple discontinuous regions spanning the entire extracellular domain (19), and perhaps the extracellular loops of the transmembrane domains, of the TSH receptor (18–21). The mutation described in our study would lead to a threonine for proline shift in amino acid residue 52. It is possible that the substitution of threonine (a neutral and polar amino acid) for proline (a neutral and hydrophobic amino acid) would result in a conformational change in the extracellular portion of the TSH receptor. Such a change could alter the immunogenicity or function of this protein.

The finding of mRNA for the TSH receptor in fibroblasts using PCR does not guarantee the presence of functional or immunologically active TSH receptor protein in these cells. Indirect evidence for the presence of this receptor on fibroblasts is suggested by our finding of enhanced expression of adhesion molecules (22) and a 72-kilodalton heat shock protein (23) after treatment of human retroocular fibroblast cultures with immunoglobulin G derived from patients with GO and high TSI levels. More direct evidence has been forwarded by Burch and colleagues (24), who demonstrated binding of polyclonal rabbit antiserum directed against a TSH receptor peptide (amino acid residues 352–367) in cultured retroocular fibroblasts. Reversible low affinity binding of [¹²⁵I]bovine TSH has been detected on porcine orbital connective tissue membranes (25).

Acropachy is a rare connective tissue manifestation of Graves' disease. Clinical features include drumstick clubbing of the digits, reticular subperiosteal new bone formation, and soft tissue swelling that is similar histologically to that seen in pretibial skin in PTD (4). In most instances, the peripheral manifestations of Graves' disease develop in hierarchy; GO is the first presentation, PTD develops next, and finally, acropachy may occur when the disease is quite prolonged or intense. It is of interest that both patients found to have the codon 52 mutation also have PTD and acropachy. In addition, these patients had among the highest TSI levels measured in our study. We hypothesize that these two patients may have a particularly intense autoimmune response directed against their TSH receptor, leading to the high TSI levels and remarkable clinical features. It may be that their mutant form of the TSH receptor is particularly immunogenic.

This is the first report, to our knowledge, of a germline mutation in the TSH receptor. Point mutations causing amino acid substitutions in membrane proteins have been causally implicated in the pathogenesis of autosomal dominant diseases, including Charcot-Marie-Tooth disease type IA (26) and hyperkalemic periodic paralysis (27). In the latter disease, mutations affecting transmembrane domains of a skeletal muscle sodium channel gene product have been identified, the presence of which presumably alters sodium channel properties leading to the disease. However, the mechanism by which a point mutation with a resulting amino acid substitution might lead to an autoimmune disease is unclear, and there exists no precedent in other autoimmune diseases.

Although various HLA-DR antigens (especially HLA-DR3) are associated with GO (28), no particular gene has been shown to confer strong susceptibility for the condition. Evidence suggests that a permissive genetic background, in addition to the presence of certain environmental factors, may be necessary for the full clinical expression of GO in patients with Graves' disease. It may be that the presence of the codon 52 mutation along with particular HLA haplotypes and environmental factors leads to an especially intense autoimmune reaction directed against TSH receptors. This reaction may be appreciated clinically as severe connective tissue involvement in Graves' disease. It is of note that neither of the patients found to have the mutation related a family history of thyroid disease. Further, two of the patients studied (patients 5 and 22) had PTD and acropachy, but did not have a TSH receptor extracellular domain mutation. Perhaps the codon 52 mutation is only one of several permissive factors involved in the development of the severe connective tissue manifestations of Graves' disease. An understanding of the potential pathogenic importance of this mutation awaits further study.

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