

## A New Graves Disease–Susceptibility Locus Maps To Chromosome 20q11.2

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### Summary

The autoimmune thyroid diseases (AITDs) include two related disorders, Graves disease (GD) and Hashimoto thyroiditis, in which perturbations of immune regulation result in an immune attack on the thyroid gland. The AITDs are multifactorial and develop in genetically susceptible individuals. However, the genes responsible for this susceptibility remain unknown. Recently, we initiated a whole-genome linkage study of patients with AITD, in order to identify their susceptibility genes. We studied a data set of 53 multiplex, multigenerational AITD families (323 individuals), using highly polymorphic and densely spaced microsatellite markers (inter-marker distance <10 cM). Linkage analysis was performed by use of two-point and multipoint parametric methods (classic LOD-score analysis). While studying chromosome 20, we found a locus on chromosome 20q11.2 that was strongly linked to GD. A maximum two-point LOD score of 3.2 was obtained at marker D20S195, assuming a recessive mode of inheritance and a penetrance of .3. The maximum nonparametric LOD score was 2.4 ( $P = .00043$ ); this score also was obtained at marker D20S195. Multipoint linkage analysis yielded a maximum LOD score of 3.5 for a 6-cM interval between markers D20S195 and D20S107. There was no evidence for heterogeneity in our sample. In our view, these results indicate strong evidence for linkage and suggest the presence of a major GD-susceptibility gene on chromosome 20q11.2.

### Introduction

The autoimmune thyroid diseases (AITDs) include two related disorders, Graves disease (GD) and Hashimoto thyroiditis (HT), in which perturbations of immune regulation result in an immune attack on the thyroid gland. In GD the autoimmune process results in the production of thyroid-stimulating antibodies and leads to hyperthyroidism, whereas in HT the end result is destruction of thyroid cells and hypothyroidism (reviewed in Davies 1996). The pathogenesis of the AITDs involves a complex interaction between genetic predisposing factors (for a review, see Tomer et al. 1997a) and environmental triggering factors (Tomer and Davies 1993). Indeed, epidemiological evidence for a genetic predisposition to the AITDs is abundant: (1) the AITDs cluster in families (Mather et al. 1980), giving a sibling risk ratio ( $\lambda_s$ ) of >15 (Vyse and Todd 1996); (2) a high concordance rate has been reported for MZ twins, compared with that for DZ twins (Harvald and Hauge 1956; Brix et al. 1998); (3) AITD is 5–10 × more common in females than in males (Volpe 1985); and (4) thyroid autoantibodies, which are markers of subclinical AITD, have been reported in up to 50% of siblings of patients with AITD (Burek et al. 1982). However, the genes causing the AITDs have not been identified. Several candidate genes have been examined previously for a possible contribution to genetic susceptibility to the AITDs, by means of both association and linkage analyses. These have included the human leukocyte antigen (HLA) genes (Payami et al. 1989; Yanagawa et al. 1993), as well as the genes for the immunoglobulin heavy chain (Nakao et al. 1980), T-cell receptor (Pickerill et al. 1993), interleukin (IL)–1 receptor antagonist (Cuddihy and Bahn 1996), thyroid stimulating hormone (TSH) receptor (De Roux et al. 1996), thyroid peroxidase (TPO; Pirro et al. 1995), and cytotoxic T lymphocyte associated-4 (CTLA-4; Yanagawa et al. 1995) (for a review, see Tomer et al. 1997a). However, with the exception of the HLA and CTLA-4 loci, which show association but not linkage, all other

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candidate genes examined gave either negative or equivocal results.

The association of the HLA genes with the AITDs has received much attention. Caucasian patients with GD have been shown to have increased HLA-DR3 (Manglabruks et al. 1991) and HLA-DQA1\*0501 haplotypes (Yanagawa et al. 1993; Barlow et al. 1996), giving relative risks of 2.0–5.0 (Farid et al. 1980; Kendall-Taylor et al. 1988; Yanagawa et al. 1993). However, our studies (Roman et al. 1992; O'Connor et al. 1993) and those of others (Shields et al. 1994) have shown no linkage between GD and the HLA region. This must mean that the HLA genes make only a minor contribution to overall genetic susceptibility to GD (Greenberg 1993; Barbesino et al. 1998a). Outside the HLA region, only the CTLA-4 gene has been found to be consistently associated with the AITDs, giving a relative risk of 2.0 (Yanagawa et al. 1995; Nistico et al. 1996). Again, we have not been able to demonstrate linkage between the CTLA-4 locus and the AITDs (Barbesino et al. 1998a), suggesting that the CTLA-4 gene also makes only a minor contribution to the overall susceptibility to the AITDs.

We recently initiated a whole-genome linkage study of families with AITD, in order to map the susceptibility genes for GD and HT. Thus far, two loci have been found to be linked with GD. The first locus (GD-1) was mapped to chromosome 14q31, ~20 cM telomeric to the TSH-receptor gene, giving a maximum LOD score of 2.5 for marker D14S81 (Tomer et al. 1997b). A second GD-linked locus was identified on chromosome Xq21.33, with marker DXS8020, giving a maximum LOD score of 2.5 (Barbesino et al. 1998b). These two loci were not linked with HT or with the AITDs as a whole (Tomer et al. 1997b; Barbesino et al. 1998b). We report the identification of a major susceptibility locus for GD, on chromosome 20q11.2, giving a maximum LOD score of 3.5.

## Patients and Methods

### Family Recruitment

This project was approved by the Mount Sinai School of Medicine institutional review board. Fifty-three families (323 individuals) were analyzed in the study (26 from the United States, 8 from Italy, 10 from Israel, and 9 from the United Kingdom). All families enrolled in the study were multiplex (more than one affected) for AITD and multigenerational. Families were ascertained through a proband with AITD who confirmed having at least one other first-degree relative with AITD. Although as many relatives as possible were recruited from each family, the *minimum* requirement for participation in the study was a family consisting of four first-degree relatives (including the proband) from two generations.

On average, our families had 6.2 members. The AITDs studied included GD and HT. GD was diagnosed by (1) documented clinical and biochemical hyperthyroidism requiring treatment, (2) a diffuse goiter, (3) the presence of TSH-receptor antibodies, and/or (4) diffusely increased <sup>131</sup>I (iodine-131) uptake in the thyroid gland. HT was diagnosed by (1) documented clinical and biochemical hypothyroidism requiring thyroid-hormone replacement and (2) the presence of autoantibodies to TPO and to thyroglobulin. For this study, all other family members, whether thyroid-autoantibody positive or negative, were classified as unaffected. For all subjects, phenotype was determined with the clinician blinded to the individual's genotype. Each participant was interviewed and examined and gave written informed consent before participating. All the pertinent clinical and laboratory data were recorded and stored in our database. At the time of each patient's interview, blood was collected for DNA purification, as well as for thyroid-function tests and thyroid-antibody testing. Anti-thyroglobulin and anti-TPO antibodies were measured by specific radioimmunoassay (Kronus).

### PCR Amplification of Microsatellite Markers

DNA was extracted from whole blood, as described elsewhere (Miller et al. 1988). Microsatellite markers were selected from the Génethon linkage maps (Dib et al. 1996) and were analyzed according to the method of Weber (1990). Oligonucleotides for amplification of the microsatellites were designed according to published sequences in the Genome Database. Fluorescent-labeled primers were purchased from Applied Biosystems. PCR was performed in 15- $\mu$ l reaction volumes containing 50 ng genomic DNA; 5 pmol each primer (one of which was fluorescent labeled); and PCR buffer containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each of dATP, dGTP, dTTP, and dCTP, and 1 U AmpliTaq DNA polymerase (Perkin Elmer Applied Biosystems). Reaction mixtures were heated to 94°C for 7 min and then were cycled 30  $\times$  as follows: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The PCR products were diluted 1:20 in double-distilled H<sub>2</sub>O and were pooled. Two microliters of the pooled products was mixed with 0.5  $\mu$ l internal size standard and 10  $\mu$ l deionized formamide and then was denatured and separated by use of an ABI 310 genetic analyzer (Applied Biosystems). Allele calling was performed by use of GENOTYPER 2.0 software. The marker data then were exported automatically to an INGRES database, for linkage analysis.

### Linkage Analysis

Linkage analysis was performed by use of parametric methods of likelihood maximization (classic LOD-score

**Table 1****Characteristics of the Current Study Sample**

Group	No. (%) of Females	No. (%) of Males	Total	F:M
Affected:				
GD	49 (81.7)	11 (18.3)	60	4.5:1
HT	72 (92.3)	6 (7.7)	78	12:1
Total	121 (87.7)	17 (12.3)	138	7.1:1
Unaffected	83 (44.9)	102 (55.1)	185	.8:1
Total	204 (63.2)	119 (36.8)	323	1.7:1

analysis). Two-point LOD scores for the different markers studied were computed by use of LIPED software (Ott 1976). Multipoint LOD scores were calculated by use of the GENEHUNTER program (Kruglyak et al. 1996). Two-point LOD scores were computed for dominant and recessive models, for 30% penetrance and over a range of recombination fractions ( $\theta$ s). We chose 30% penetrance on the basis of the most recent twin data (Brix et al. 1998). In addition, the nonparametric LOD (NPL) scores were computed by use of the GENEHUNTER program. In the largest epidemiological survey of thyroid diseases, the prevalence of AITD was up to ~1% (Tunbridge et al. 1977; Vanderpump et al. 1995). We, therefore, assumed that the disease prevalence was .01 and adjusted the gene frequency according to the model used (dominant or recessive) and the assumed penetrance (30%), assuming Hardy-Weinberg equilibrium. For multipoint analyses, we used the recessive model only and, again, assumed 30% penetrance. Multipoint LOD scores were computed for the entire chromosome 20, by use of 13 markers (see Results).

**Models**

At present it is not clear whether GD and HT are two distinct disorders with different etiologies or whether common etiologic factors are involved in the pathogenesis of these disorders. Indeed, both disorders can occur in the same family, and 34% of the families in our data set included first-degree relatives with GD and HT. Therefore, we analyzed the data by using three models: (1) all AITD patients were considered affected, (2) only GD patients were considered affected (under this model, HT patients were considered unaffected, even if they had relatives with GD), and (3) only HT patients were considered affected (under this model, GD patients were considered unaffected, even if they had relatives with HT). Family members with thyroid autoantibodies only were classified as unaffected. In addition, we tested the data set for heterogeneity. Heterogeneity testing was performed by use of GENEHUNTER (Kruglyak et al. 1996).

**Power Calculations**

Simulation studies were performed to assess (1) the power of our 53 families to detect linkage and (2) the maximum attainable LOD scores for our data set. We assumed a penetrance of 30% for our simulations, on the basis of the reported 30% concordance rate in MZ twins (Brix et al. 1998). The simulation software used for the power calculations (Greenberg and Berger 1994) generated 53 families that were homologous to the families that we included in our study. On the basis of the prevalence of the AITDs in the general population (1%; Vanderpump et al. 1995) and the assumed 30% penetrance, we calculated the frequency of a disease-linked gene (assuming Hardy-Weinberg equilibrium) to be .067 for dominant inheritance and .4 for recessive inheritance. The simulations demonstrated that use of a data set of 53 families gave statistical power to reject linkage, within a 10 cM-region ( $\theta = .1$ ), at a penetrance of .3. Thus, our families were sufficient to reject linkage to the tested markers. Simulations also showed that we had the power to detect linkage at a penetrance of .3, using the 53 families. The maximum LOD scores were >4.0 for data generated with a marker at  $\theta = .01$  units from the disease gene and >2.8 for a marker at  $\theta = .05$  units from the disease gene. The theoretical maximum LOD score attainable for our data set, assuming a recessive model and  $\theta = .01$ , was 6.7.

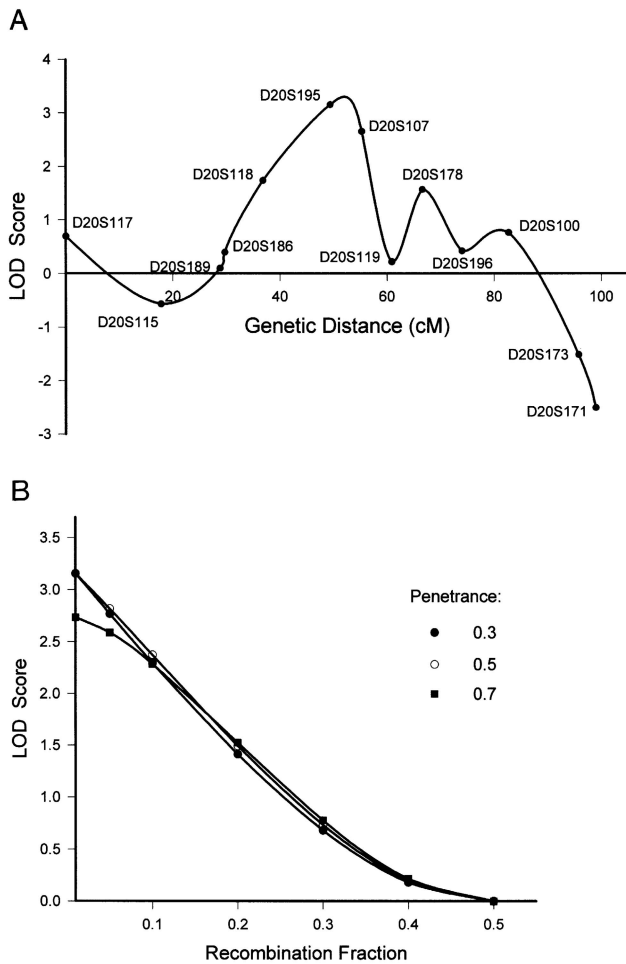
**Results****Characteristics of the Study Sample**

Tables 1 and 2 show the clinical characteristics of the 53 families studied. Of the 53 families, 14 (26%) had GD-affected members only, 21 (40%) had HT-affected members only, and 18 (34%) had both GD- and HT-affected first-degree relatives. Of the 138 affected individuals, 121 (87.7%) were female, and the female:male (F:M) ratio for affected family members (7.1:1) was in accordance with that reported in the literature (Volpe 1985). Of the clinically and biochemically unaffected family members, 35% had thyroid antibodies, which is similar to the incidence reported in previous studies (Burek et al. 1982; Aho et al. 1983). Interestingly, the F:M ratio for the thyroid antibody-positive unaffected individuals was 1:1, which is much lower than the F:M

**Table 2****Thyroid-Antibody Status of the Unaffected Family Members**

Thyroid-Antibody Status	Percentage of Unaffecteds <sup>a</sup>	F:M
Positive	35	1:1
Negative	65	.8:1

<sup>a</sup>  $n = 185$ ; F:M = .8:1.



**Figure 1** A, Maximum two-point LOD-score results for markers on chromosome 20, at  $\theta = .01$ , when patients with GD were classified as affected. The highest LOD score was obtained for marker D20S195 (Maximum LOD Score = 3.2), and the LOD scores for other markers in the region were positive in a geographically logical sequence. B, LOD scores for marker D20S195, under the recessive model, at three penetrances (.3, .5, and .7) and different  $\theta$ s. The maximum LOD score was 3.2, obtained at  $\theta = .01$ .

ratio for the affected family members, suggesting different pathogenic mechanisms for the development of the AITDs and the propensity to secrete thyroid autoantibodies.

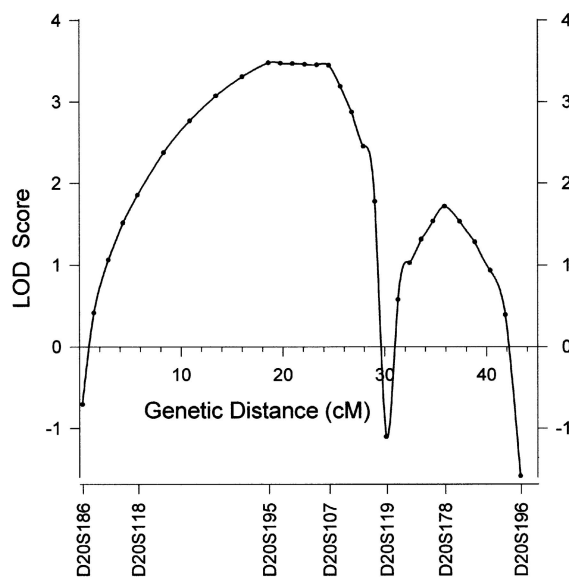
*Screening for Loci Linked with the AITDs, GD, and HT*

Analysis of microsatellite markers on chromosome 20 showed no evidence of linkage with either HT or with the AITDs as a whole (data not shown). However, when the data for GD affected status were analyzed, highly positive LOD scores were obtained for chromosome 20q11.2 (fig. 1A). The maximum two-point LOD score was 3.2 for marker D20S195, obtained for the recessive model, at a penetrance of 30% and  $\theta = .01$ . Moreover, the LOD scores for other markers in the local region of

D20S195 were positive in a geographically logical sequence (fig. 1A). At this point we decided to test the effect, on our linkage analysis, of varying the penetrance, and we reanalyzed the data for the marker giving the highest LOD score (D20S195), for three different penetrances (.3, .5, and .7). The results showed that in our analysis variation of the penetrance did not change the LOD score significantly, further supporting the evidence for linkage to D20S195 (fig. 1B).

*Multipoint LOD-Score Analysis*

For the multipoint analysis, we used a genetic map for chromosome 20, with sex-averaged distances between markers (in  $\theta$  units) as follows: D20S117-.178-D20S115-.11-D20S189-.009-D20S186-.071-D20S118-.126-D20S195-.059-D20S107-.056-D20S119-.057-D20S178-.074-D20S196-.087-D20S100-.131-D20S173-.032-D20S171. This order and the  $\theta$  units were obtained from the Génethon maps (Dib et al. 1996) and were verified with our data set. Multipoint linkage analysis localized the new GD-susceptibility locus on chromosome 20 to within an interval of ~6 cM between markers D20S195 and D20S107. The multipoint LOD scores throughout this interval were 3.5 (fig. 2). Further testing showed no evidence for heterogeneity in our data set ( $\alpha = 1.0$ ; maximum heterogeneity LOD score of 3.5). Examination of individual families showed that 80% of the families had positive LOD scores for marker D20S195, within the range 0.1–0.4.



**Figure 2** Multipoint LOD-score analysis for the region 20q11-12. The multipoint linkage analysis localized the new GD-susceptibility locus to within an interval of ~6 cM between markers D20S195 and D20S107. The multipoint LOD scores throughout this interval were 3.5.

These results further support lack of heterogeneity in our data set.

#### NPL-Score Analysis

NPL-score analyses were performed by use of the GENEHUNTER program. The maximum multipoint NPL score was obtained for marker D20S195, the same marker that gave the highest parametric LOD score. The maximum multipoint NPL score was 2.4 ( $P = .00043$ ), which supports the evidence for linkage at that locus.

#### Influence of HT in GD Families

Our GD families consisted of some with the GD phenotype only and some with both GD and HT phenotypes (mixed families). In order to examine whether the genetic influence of the GD-susceptibility locus was different in the group of GD families in which some family members had HT, we tested these families separately. In this analysis, the HT individuals in the mixed families were classified as unaffected. Subgrouping the GD families into those in which HT did and did not appear demonstrated that the LOD scores for marker D20S195, which gave the highest two-point LOD score, were positive for both subsets of families, contributing approximately equally to the total LOD score (table 3). This suggested that the contribution of the GD-susceptibility locus to the susceptibility to GD was similar in all families with GD that we examined, whether they included only GD-affected members or both GD- and HT-affected members. Subgrouping of our HT families into HT-only families and families with both GD and HT (mixed families) did not change the negative linkage results obtained for HT. In this analysis, the GD patients in the mixed families were classified as unaffected. For both groups of families the LOD scores were negative for all markers on chromosome 20 (data not shown).

#### Influence of Country of Origin on GD Families

The GD families also were subgrouped by their country of origin. After subgrouping the GD families, the LOD scores obtained for marker D20S195 demonstrated that both the North American families and the European families contributed approximately equally to the total LOD score (table 3). This suggested that the contribution of the new GD-susceptibility locus to the susceptibility to GD was similar in the North American and the European families with GD that we examined, again supporting the evidence for no heterogeneity in our data set.

#### Discussion

We report the identification of a new GD-susceptibility locus on chromosome 20q11.2, giving a maximum LOD score of 3.5, which represents strong evidence in favor

**Table 3**

**LOD Scores for Marker D20S195, for Different Subsets of the GD Families**

Family Subset	Penetrance	$\theta$	LOD Score
By affected status:			
GD only	.3	.01	1.5
Mixed (GD+HT) <sup>a</sup>	.3	.01	1.7
All	.3	.01	3.2
By country of origin:			
North American	.3	.01	1.6
European	.3	.01	1.6
All	.3	.01	3.2

<sup>a</sup> Patients with HT were classified as unaffected, for the GD analysis.

of linkage (Thomson 1994; Lander and Kruglyak 1995). The fact that in our analysis variation of the penetrances did not significantly alter the results (fig. 1B) shows that the results are relatively insensitive to model details and further supports the evidence for a linked locus at chromosome 20q11.2. The new GD-susceptibility locus is not linked to HT, since analysis of the data for the HT families resulted in strongly negative LOD scores. Moreover, in families with GD- and HT-affected individuals, the gene was specific only to the GD-affected individuals, demonstrating its high specificity for GD.

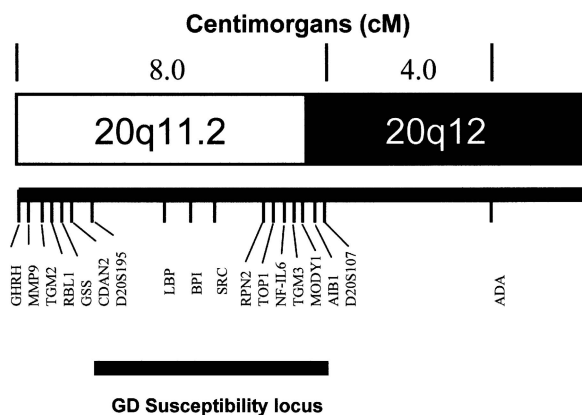
Whereas the major histocompatibility complex (MHC) genes have shown the strongest *association* with GD, giving a relative risk of up to 5 (Farid et al. 1980), several studies have shown evidence against linkage of the HLA region to GD (Roman et al. 1992; O'Connor et al. 1993; Shields et al. 1994). This suggests that the HLA genes confer only a minor, modulating effect on the overall susceptibility to GD and that the major genes contributing to the susceptibility to GD are located outside the MHC region. Additional support for this conclusion has been provided by the study of animal models. An animal model of GD was developed recently by Shimojo et al. (1996) and has been extended in our laboratory (Kita et al. 1997). In this model, autoimmune hyperthyroidism was induced in H-2<sup>k</sup> mice, by immunization with TSH receptor-transfected fibroblasts spontaneously expressing MHC class I and class II antigens. Since all the mice had the same MHC and were exposed to identical environmental conditions, we expected 100% of the mice to develop the disease if susceptibility were dependent only on MHC-mediated genetic influences. However, consistently only 25%–50% of the immunized mice developed the disease. This suggested non-MHC genetic influences, since all the animals had identical MHC genes. Indeed, the susceptibility of the mice to the induction of GD was shown to be influenced by non-MHC genes, when the results for mouse strains that share the H-2<sup>k</sup> haplotype but differ in their genetic background were compared (Yamaguchi et al. 1997).

As with all complex and common diseases, the genetic

susceptibility to AITD lacks a simple Mendelian pattern of inheritance and probably involves several genes with varying penetrances and interactions. Thus, the identification of susceptibility genes for complex diseases is very difficult. One possible solution is to test for linkage by use of different models. We tested our markers for linkage with AITD by using two models, dominant and recessive. In addition, we decided a priori to subgroup the AITDs into HT and GD, on the basis of clinical criteria, and to test each subgroup independently as well as to test the AITDs as a whole. Although these multiple analyses enabled us to maximize the LOD score, with respect to the model and the phenotype of the disease, they may have weakened the evidence for linkage, because of multiple testing. It recently was suggested that, when dominant and recessive models are used, the maximum LOD score obtained should be reduced by 0.3 (Hodge et al. 1997). With regard to our study, this would result in a maximum LOD score of 3.2, which still strongly supports the evidence in favor of linkage (Lander and Kruglyak 1995). When the data were analyzed by use of the nonparametric approach, which does not assume a mode of inheritance, the maximum LOD score was 2.4 ( $P = .00043$ ), further supporting the evidence for linkage. The lower LOD score obtained with the nonparametric analysis was expected, in view of the loss of information that is associated with nonparametric methods of analysis. Thus, analyzing for two genetic models did not significantly change the strength of our linkage results and enabled us to find the model that best approximated the inheritance of the susceptibility locus on chromosome 20.

One of the most important confounding factors in the mapping of complex disease genes has been heterogeneity. The existence of genetic heterogeneity within a data set can mask true genes conferring susceptibility only to a subset of the data set. With regard to this new GD-susceptibility locus on chromosome 20, our individual family analyses showed no evidence for heterogeneity, and the newly mapped GD locus was linked in the majority of our GD families. This conclusion was supported by the statistical analyses of the data, which also failed to show evidence for heterogeneity in our data set. Moreover, when we subgrouped our families according to clinical subsets of GD (GD-only families and mixed families) and country of origin (North American and European families), the results also showed no evidence for heterogeneity.

The location of the new GD-susceptibility locus was determined to be in a 6-cM interval between markers D20S195 and D20S107. This region contains at least 17 known human genes (fig. 3). Examination of the genes mapped to this region showed that all except one were not known to encode for proteins related to immune or thyroid functions. However, IL-6 nuclear factor



**Figure 3** Linkage map of human chromosome 20q11.2-20q12. This region is ~12 cM long, and at least 17 human genes have been mapped to this region. The relative positions of the markers spanning the region of the new GD-susceptibility locus are indicated, with the genetic distances given in centimorgans. Of the known genes in this region, only NF-IL6 may be a candidate gene for GD, since it is involved in the control of inflammatory responses.

(NF-IL6) encodes a protein that, theoretically, may play a role in the development of GD. NF-IL6 is a transcription protein, localized to the nucleus, that binds to an IL-1 response element in the IL-6 gene and to regulatory regions of other inflammation-associated and cytokine genes. It is believed to play a role in the regulation of acute-phase reactions and inflammation. Identification of polymorphisms in the NF-IL6 gene associated with GD may determine whether this gene is the new GD-susceptibility locus. Alternatively, the chromosome 20q11.2 region may contain a currently unidentified GD-causing gene or genes.

In summary, our data demonstrate that a gene locus conferring susceptibility to GD was localized to chromosome 20q11.2 and provided a framework for further localization of the gene. This locus appears to predispose to GD in both GD-only families and in mixed families with GD- and HT-affected members. The role of this locus in GD patients with no family history of AITD remains to be determined. Identification of the new GD-susceptibility locus will require a multifaceted approach, including analysis of candidate genes in the region (e.g., NF-IL6) and positional cloning. Identification of the new GD gene will provide important information on the genetic mechanisms predisposing to the development of GD.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Généthon, <http://www.genethon.fr> (for linkage maps of microsatellite markers used in PCR amplification)  
Genome Database, <http://gdbwww.gdb.org> (for sequences of oligonucleotides used in PCR amplification)

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