Molecular Genetics and Metabolism **70,** 214–218 (2000)
doi:10.1006/mgme.2000.3007, available online at http://www.idealibrary.com on IDEAL®

Complex Association Analysis of Graves Disease Using a Set of Polymorphic Markers

Dimitry A. Chistyakov,*[,1] Kirill V. Savost'anov,* Rustam I. Turakulov,* Natal'ya A. Petunina,† Lyubov' V. Trukhina,† Anna V. Kudinova,† Mikhail I. Balabolkin,‡ and Valery V. Nosikov†

**Department of Molecular Diagnostics, State Research Center GosNIIgenetika, 1st Dorozhny Proezd 1, Moscow 113545, Russia; ‡Endocrinology Research Center, Dm. Ul'yanov st. 11, Moscow 117036, Russia; and †Department of Endocrinology and Diabetology, I. M. Sechenov's Medical Academy, Gen. Glagolev st. 19, Moscow 119435, Russia*

Received April 21, 2000, and in revised form May 19, 2000

Graves disease is complex autoimmune thyrotoxicosis. A number of genes may contribute to the development of the disorder. Some of them may be genes that encode cytotoxic T-lymphocyte-associated serine esterase-4 (CTLA4), subunit 2 of large multifunctional protease (LMP2), thyroid-stimulating hormone receptor (TSHR), and interleukin 1 receptor antagonist (IL1RN). We studied polymorphism of Ala17Thr CTLA4, H60R LMP2, Pro52Thr TSHR, and IL1RN–VNTR in healthy controls (n = 93) and patients with Graves disease (n = 78) using PCR. To study CTLA4, H60R, and TSHR polymorphism, PCR products were digested with *Mbo*I, *Hin*6I and *Psy*I, respectively. Comparative analysis using χ^2 test showed significant differences in allele and genotype frequency of Ala17Thr polymorphic marker between the two groups studied. Thus, the CTLA4 gene may be involved in the pathogenesis of Graves disease in a Moscow population. © 2000 Academic Press

Key Words: **CTLA4; LMP2; TSHR; IL1RN; association; Graves disease.**

Graves disease is autoimmune thyroid syndrome. A number of genes are known to be involved in the development of this disorder. One of them, encoding for cytotoxic T-lymphocyte-associated serine esterase-4 (CTLA4), is a common marker of many autoimmune disorders such as insulin-dependent diabetes mellitus (IDDM), Graves disease, rheumatoid arthritis, and others (1,2). A-to-G substitution at nucleotide 49 of the CTLA4 gene exon 1 results in an amino acid exchange (Thr/Ala) at codon 17 in the leader peptide of the expressed protein (3). Alanine at codon 17 of CTLA4 is shown to be associated with genetic susceptibility to Graves disease as well as to IDDM (2).

The LMP2 gene encodes one of the subunits of multifunctional proteasome that generates peptides from cytosolic proteins for transport into the endoplasmic reticulum where they associate with major histocompatibility complex (MHC) class I molecules. The gene is located in the MHC class II region (4). Histidine-to-arginine (H60R) dimorphism was identified at position 60, and the R allele was shown to be associated with high risk of Graves disease (5). The R allele is observed to be in linkage disequilibrium with associated HLA DRB1*0304-DQB1*02-DQA1*0501 haplotype, also a strong risk marker of Graves disease (5).

Molecules of thyroid-stimulating hormone receptor (TSHR) on a surface of thyrocytes are shown to be a major target for autoantibodies in patients with Graves disease. However, evidence of a connection between the TSHR locus (14q31) and susceptibility to the disease are controversial (6,7) as are results of association studies of Pro52Thr polymorphism at the gene (8,9).

Development of autoimmune thyrotoxicosis is often accompanied by inflammatory events that are directed by interleukin 1 (IL1) and other cytokines. The IL1RN gene encodes an antagonist protein that

[1] To whom correspondence should be addressed at Department of Molecular Diagnostics, State Research Center GosNIIgenetika, 1st Dorozhny Proezd, Building 1, Moscow 113545, Russia. Fax: (7095) 315 05 01; e-mail: dimitry.chistiakov@eudoramail.com.

1096-7192/00 $35.00

binds to IL1 receptors and inhibits the binding of IL1-α and IL1-β. A variable number of tandem repeats (VNTR) is located at intron 2 and consists of five alleles (10). One of them containing two 86-bp tandem repeats is shown to be related to the pathogenesis of a number of inflammatory and autoimmune disease (11–14).

Thus, all four genes may be considered as candidates in Graves disease. In this article we evaluate polymorphic markers at the genes for association with the disorder in a Moscow population.

MATERIALS AND METHODS

Patients

We studied 78 patients with Graves disease (M/F ratio 14/64, age 39.3 ± 11.6 years, duration of the disease 4.1 ± 2.8 years) and 93 healthy controls (M/F ratio 30/63, age 36.5 ± 12.5 years). Control subjects have no autoimmune, cardiovascular, or other disorders.

DNA Analysis

DNA was extracted from whole human blood with phenol and chloroform (15). Polymorphic regions were amplified by polymerase chain reaction (PCR) in 50 μl of the reaction mixture containing 100 ng of genomic DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 2.5 units of *Taq* polymerase (Biotekh, Russia), and buffer. To amplify polymorphic DNA fragments of CTLA4, LMP2, and TSHR genes, we used PCR buffer consisting of 10 mM Tris–HCl (pH 8.8), 50 mM potassium chloride, 1.5 mM magnesium chloride, and 10% dimethyl sulfoxide, and primers whose sequence is described by Marron *et al.* (3), Deng *et al.* (16), and De Roux *et al.* (17), respectively. For ILR1N-VNTR, PCR primers described by Tarlow *et al.* (10) and buffer containing 67 mM Tris–HCl (pH 8.8), 16.7 mM ammonium chloride, 1.0 mM magnesium chloride, and 0.1% twin 20, were added into the reaction mixture. PCR was run in a PHC-2 thermal cycler (Techne, UK) and included 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C (IL1RN), 60°C (CTLA4 and LMP2), or 65°C (TSHR), and extension for 1 min at 72°C.

Amplified fragments of CTLA4, LMP2, and TSHR were digested with *Mbo*I, *Hin*6I, and *Psy*I (all enzymes from Fermentas, Lithuania), respectively. DNA products after PCR amplification and digestion were separated in 2% agarose gel and visualized by ethidium bromide staining.

TABLE 1
Allele and Genotype Distributions of Ala17Thr CTLA4 Gene in Healthy Donors and Patients with Graves Disease

	Control *n* (%)	Graves *n* (%)	RR
	Allele distribution		
Ala	98 (52.7)	122 (78.2)	3.19
Thr	88 (47.3)	34 (21.8)	0.31
χ^2 (df)		24.073 (1)	
P		<0.001	
	Genotype distribution		
Ala/Ala	30 (32.3)	50 (64.1)	3.69
Ala/Thr	38 (40.9)	22 (28.2)	0.57
Thr/Thr	25 (26.9)	6 (7.7)	0.24
χ^2 (df)		19.748 (2)	
P		<0.001	

Statistical Analysis

Genotype frequencies were checked for deviation from the Hardy–Weinberg equilibrium by the χ^2 and *G*-statistic tests, using the Rows and Columns program based on the Roff and Bentzen algorithm (18). Genotype and allele frequencies in the groups studied were compared by χ^2 analysis. A difference was considered significant at $P < 0.05$. The relative risk (*RR*) was determined as described by Thomson (19).

RESULTS

Ala17Thr Polymorphism of the CTLA4 Gene

Frequency of both alleles was found to be approximately equal in healthy donors (Table 1). Ala/Thr heterozygotes (41%) were the most common. Among genotypes the observed genotype frequencies obeyed the Hardy–Weinberg equilibrium ($\chi^2 = 1.3954$ and *G*-statistic = 1.3974, $P = 0.5490 \pm 0.0157$).

In affected patients a 2.2-fold decrease in the frequency of the Thr allele was found whereas a portion of the Ala allele was increased in comparison with healthy controls (Table 1). A 2.0-fold increase in the frequency of the Ala/Ala genotype happened in subjects with Graves disease also. At the same time two other genotypes were decreased in the patients. Changes in allele and genotype frequency were highly significant.

Ala17Thr polymorphism of the CTLA4 gene is strongly associated with Graves disease in a Moscow

TABLE 2
Allele and Genotype Distributions of H60R LMP2 Gene in Healthy Donors and Patients with Graves Disease

	Control *n* (%)		Graves *n* (%)
Allele distribution			
H	142 (76.3)		106 (67.9)
R	44 (23.7)		50 (32.1)
χ^2 (df)		3.00 (1)	
P		>0.05	
Genotype distribution			
HH	55 (59.1)		40 (51.3)
HR	32 (34.4)		26 (33.3)
RR	6 (6.5)		12 (15.4)
χ^2 (df)		3.702 (2)	
P		>0.05	

TABLE 3
Allele and Genotype Distributions of Pro52Thr TSHR Gene in Healthy Donors and Patients with Graves Disease

	Control *n* (%)		Graves *n* (%)
Allele distribution			
Pro	139 (76.3)		117 (75.0)
Thr	47 (47.4)		39 (25.0)
χ^2 (df)		0.003 (1)	
P		>0.05	
Genotype distribution			
Pro/Pro	55 59.1)		46 (59.0)
Pro/Thr	35 (34.4)		25 (32.1)
Thr/Thr	6 (6.5)		7 (9.0)
χ^2 (df)		0.654 (2)	
P		>0.05	

population. The Thr allele (RR = 0.31) and the Thr/Thr genotype (RR = 0.23) may act as protective factors while the Ala allele and homozygotes Ala/Ala in particular are associated with increased risk of autoimmune thyrotoxicosis.

H60R Polymorphism of the LMP2 Gene

The H allele and the HH genotype were the most common in the controls (Table 2). Observed heterozygosity was 34.4%. The observed genotype frequencies were in concordance with the Hardy–Weinberg equilibrium (χ^2 = 0.1607 and *G*-statistic = 0.1609, $P = 0.9370 \pm 0.0077$).

No significant difference in allele and genotype frequency of H60R marker was detected between the affected patients and the healthy controls (Table 2). Thus, the H60R mutation at the LMP2 gene is not related to Graves disease.

Pro52Thr Polymorphism of the TSHR Gene

In the controls the Pro allele was three times more frequent than the Thr allele. Pro/Pro homozygotes occured 8.6 times more frequently than the Thr/Thr genotype (Table 3). The observed heterozygosity was 37.6%. The observed genotype distribution completely obeyed the Hardy–Weinberg equilibrium (both χ^2 and *G*-statistic = 0, $P = 1.0$). No significant difference in allele and genotype distribution was found in the affected patients and the controls. Thus, Pro52Thr polymorphism of the TSHR gene is not associated with Graves disease in the Moscow population.

VNTR Polymorphism of the IL1RN Gene

Five alleles from 240 to 580 bp in length were found in the control subjects. The alleles were designated according to the number of tandem repeats. Two alleles, 2 and 4, were the most frequent (Table 4). Among genotypes, heterozygotes 2/4 and homozygotes 4/4 were the most widespread. Heterozygosity was observed to equal 0.559. The observed genotype frequencies obeyed the Hardy–Weinberg equilibrium (χ^2 = 2.8208 and *G*-statistic = 3.2251, $P = 0.9910 \pm 0.0030$).

Allele frequencies of IL1RN–VNTR were not sig-

TABLE 4
Allele Distribution of ILR1N–VNTR in Healthy Donors and Patients with Graves Disease

Allele			
Number	Length, bp	Control *n* (%)	Graves *n* (%)
2	240	52 (28.0)	60 (38.5)
3	325	6 (3.2)	3 (1.9)
4	410	115 (61.8)	88 (56.4)
5	495	7 (3.8)	3 (1.9)
6	580	6 (3.2)	2 (1.3)
χ^2 (df)		6.179 (4)	
P		>0.05	

nificantly different in the controls and the patients (Table 4). This finding suggests no association of the VNTR with the disorder.

DISCUSSION

A positive association between the Ala17Thr dimorphism of the CTLA4 gene and Graves disease was shown in a number of populations (2,20–22) except for the negative data of Djilali-Saiah *et al.* (23). Moreover, the polymorphism was found to be linked to the disorder (21). Besides the mutation at codon 17, another polymorpic marker, namely dinucleotide microsatellite at exon 3, was linked to Graves disease as well (22,24,25). Our data suggest an important role of the CTLA4 gene in the pathogenesis of the disorder. CTLA4 and HLA (human leucocyte antigens) genes may be major susceptibility loci supporting first and foremost an autoimmune origin of Graves disease.

In spite of our data, one of the non-HLA genes, LMP2, that nevertheless is located in the HLA locus, may also be involved in the disorder. This evidence is supported by our data and results obtained by Heward *et al.* (5) who have shown a relation of both the R allele and the RH genotype of the LMP2 gene to increased risk of Graves disease. It is difficult to estimate an exact contribution of the gene to the suspectibility because of linkage disequilibrium with MHC class II genes (9). Protein product of the LMP2 gene may be involved in processing of specific thyroid autoantigens followed by the induction of functional antibodies against them.

One such autoantigen may be the TSH receptor molecule. This was suggested by Shimojo *et al.* (26) who induced immune hyperthyroidism with the major humoral and histologic features of Graves disease in mice that were immunized with fibroblasts transfected with both the human TSHR and MHC class II molecule. Neighboring polymorphic markers together with microsatellite at intron 7 of the TSHR gene were shown to be linked to Graves disease (7,25). However, the mutation at codon 52 is likely to have no pathogenetic relevance in Graves disease that is suggested by our data and other studies (27,28). On the other hand, it cannot be excluded that another mutation or sequence polymorphism in the remainder of the TSHR extracellular domain might be an important autoimmune mechanism of Graves disease.

The association between VNTR in the IL1RN gene and the disorder is still questionable. Our results are not in agreement with the data of Blakemore *et al.* (13) showing the predisposing role of allele 2 in the development of Graves disease. However, studies in the United States (29) and Belgium (30) have not found any positive association yet. It is more likely that the IL1RN gene plays a minor role in the pathogenesis of autoimmune thyrotoxicosis because inflammation is not permanent in Graves disease.

REFERENCES

1. Gonzalez-Escribano MF, Rodriguez R, Valenzuela A, Garcia A, Garcia-Lozano JR, Nunez-Roldan A. CTLA4 polymorphisms in Spanish patients with rheumatoid arthritis. *Tissue Antigens* **53:**296–300, 1999.
2. Donner H, Rau H, Walfish PG, Braun J, Siegmund T, Finke R, Herwig J, Usadel KH, Badenhoop K. CTLA4 alanine-17 confers genetic susceptibility to Graves' disease and to type 1 diabetes mellitus. *J Clin Endocrinol Metab* **82:**143–146, 1997.
3. Marron MP, Raffel LJ, Garchon HJ, Jacob CO, Serrano-Rios M, Martinez Larrad MT, Teng WP, Park Y, Zhang ZX, Goldstein DR, Tao YW, Beaurain G, Bach JF, Huang HS, Luo DF, Zeidler A, Rotter JI, Yang MC, Modilevsky T, Maclaren NK, She JX. Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA4 polymorphisms in multiple ethnic groups. *Hum Mol Genet* **6:**1275–1282, 1997.
4. Kelly A, Powis SH, Glynne R, Radley E, Beck S, Trowsdale J. Second proteasome-related gene in the human MHC class II region. *Nature* **353:**667–668, 1991.
5. Heward JM, Allahabadia A, Sheppard MC, Barnett AH, Franklyn JA, Gough SC. Association of the large multifunctional proteasome (LMP2) gene with Graves' disease is a result of linkage disequilibrium with the HLA haplotype DRB1*0304-DQB1*02-DQA1*0501. *Clin Endocrinol* **51:**115–118, 1999.
6. De Roux N, Shields DC, Misrahi M, Ratanachaiyavong S, McGregor AM, Milgrom E. Analysis of the thyrotropin receptor as a candidate gene in familial Graves' disease. *J Clin Endocr Metab* **81:**3483–3486, 1996.
7. Tomer Y, Barbesino G, Keddache M, Greenberg DA, Davies TF. Mapping of a major susceptibility locus for Graves' disease (GD-1) to chromosome 14q31. *J Clin Endocr Metab* **82:**1645–1648, 1997.
8. Sunthornthepvarakul T, Hayashi Y, Refetoff S. Polymorphism of a variant human thyrotropin receptor (hTSHR) gene. *Thyroid* **4:**147–149, 1994.
9. Simanainen J, Kinch A, Westermark K, Winsa B, Bengtsson M, Schuppert F, Westermark B, Heldin NE. Analysis of mutations in exon 1 of the human thyrotropin receptor gene: High frequency of the D36H and P52T polymorphic variants. *Thyroid* **9:**7–11, 1999.
10. Tarlow JK, Blakemore AI, Lennard A, Solari R, Hughes HN, Steinkasserer A, Duff GW. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. *Hum Genet* **91:**403–404, 1993.
11. Pociot F, Ronningen KS, Bergholdt R, Lorenzen T, Johan-

nesen J, Ye K, Dinarello CA, Nerup J. Genetic susceptibility markers in Danish patients with type 1 (insulin-dependent) diabetes—Evidence for polygenicity in man. *Autoimmunity* **19:**169–178, 1994.

12. Blakemore AI, Tarlow JK, Cork MJ, Gordon C, Emery P, Duff GW. Interleukin-1 receptor antagonist gene polymorphism as a disease severity factor in systemic lupus erythematosus. *Arthritis Rheum* **37:**1380–1385, 1994.
13. Blakemore AI, Watson PF, Weetman AP, Duff GW. Association of Graves' disease with an allele of the interleukin-1 receptor antagonist gene. *J Clin Endocrinol Metab* **80:**111–115, 1995.
14. Fang XM, Schroder S, Hoeft A, Stuber F. Comparison of two polymorphisms of the interleukin-1 gene family: Interleukin-1 receptor antagonist polymorphism contributes to susceptibility to severe sepsis. *Crit Care Med* **27:**1330–1334, 1999.
15. Budowle B, Baechtel FS. Modifications to improve the effectiveness of restriction fragment length polymorphism. *Appl Electrophor* **1:** 181–187, 1990.
16. Deng GY, Muir A, Maclaren NK, She JX. Association of LMP2 and LMP7 genes within the major histocompatibility complex with insulin-dependent diabetes mellitus: Population and family studies. *Am J Hum Genet* **56:**528–534, 1995.
17. De Roux N, Shields DC, Misrahi M, Ratanachaiyavong S, McGregor AM, Milgrom E. Analysis of the thyrotropin receptor as a candidate gene in familial Graves' disease. *J Clin Endoc Metab* **81:**3483–3486, 1996.
18. Roff DA, Bentzen P. The statistical analysis of mitochondrial DNA: χ^2 and problem of small samples. *Mol Biol Evol* **6:**539–545, 1989.
19. Thomson G. A review of theoretical aspects of HLA and disease associations. *Theor Popul Biol* **20:**168–208, 1981.
20. Awata T, Kurihara S, Iitaka M, Takei S, Inoue I, Ishii C, Negishi K, Izumida T, Yoshida Y, Hagura R, Kuzuya N, Kanazawa Y, Katayama S. Association of CTLA-4 gene A-G polymorphism (IDDM12 locus) with acute-onset and insulin-depleted IDDM as well as autoimmune thyroid disease (Graves' disease and Hashimoto's thyroiditis) in the Japanese population. *Diabetes* **47:**128–129, 1998.
21. Vaidya B, Imrie H, Perros P, Young ET, Kelly WF, Carr D, Large DM, Toft AD, McCarthy MI, Kendall-Taylor P, Pearce SH. The cytotoxic T lymphocyte antigen-4 is a major Graves' disease locus. *Hum Mol Genet.* **8:**1195–1199, 1999.
22. Yanagawa T, Yoshida Y, Saito Y, Niimi H, Inoue I, Tahara K. CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population. *J Clin Endocrinol Metab* **80:**41–45, 1995.
23. Djilali-Saiah I, Larger E, Harfouch-Hammoud E, Timsit J, Clerc J, Bertin E, Assan R, Boitard C, Bach JF, Caillat-Zucman S. No major role for the CTLA-4 gene in the association of autoimmune thyroid disease with IDDM. *Diabetes* **47:**125–127, 1998.
24. Kotsa K, Watson PF, Weetman AP. A CTLA-4 gene polymorphism is associated with both Graves disease and autoimmune hypothyroidism. *Clin Endocrinol* **46:**551–554, 1997.
25. Sale MM, Akamizu T, Howard TD, Yokota T, Nakao K, Mori T, Iwasaki H, Rich SS, Jennings-Gee JE, Yamada M, Bowden DW. Association of autoimmune thyroid disease with a microsatellite marker for the thyrotropin receptor gene and CTLA-4 in a Japanese population. *Proc Assoc Am Physicians* **109:**453–461, 1997.
26. Shimojo N, Kohno Y, Yamaguchi K, Kikuoka S, Hoshioka A, Niimi H, Hirai A, Tamura Y, Saito Y, Kohn LD, Tahara K. Induction of Graves-like disease in mice by immunization with fibroblasts transfected with the thyrotropin receptor and a class II molecule. *Proc Natl Acad Sci* **93:**11074–11079, 1996.
27. Kaczur V, Szalai C, Falus A, Nagy Z, Krajczar G, Balazs C. Polymorphism of the 52 triplet gene (nucleotide 253) of the TSH receptor in Basedow-Graves patients and in healthy controls. *Orv Hetil* **138:**1625–1628, 1997.
28. Pearce SH, Foster DJ, Imrie H, Myerscough N, Beckett GJ, Thoday KL, Kendall-Taylor P. Mutational analysis of the thyrotropin receptor gene in sporadic and familial thyrotoxicosis. *Thyroid* **7:**923–927, 1997.
29. Cuddihy RM, Bahn RS. Lack of an association between alleles of interleukin-1 alpha and interleukin-1 receptor antagonist genes and Graves' disease in a North American Caucasian population. *J Clin Endocrinol Metab* **81:**4476–4478, 1996.
30. Muhlberg T, Kirchberger M, Spitzweg C, Herrmann F, Heberling HJ, Heufelder ARE. Lack of association of Graves' disease with the A2 allele of the interleukin-1 receptor antagonist gene in a white European population. *Eur J Endocrinol* **138:**686–690, 1998.