

# Genotype and Phenotype Predictors of Relapse of Graves' Disease after Antithyroid Drug Withdrawal

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## Key Words

Graves' disease · Costimulatory gene · CTLA-4 · CD40 · Smoking · Relapse predictors

## Abstract

**Background:** For patients with Graves' disease (GD), the primary goal of antithyroid drug therapy is to temporarily restore the patient to the euthyroid state and wait for a subsequent remission of the disease. This study sought to identify the predictive markers for the relapse of disease. **Methods:** To do this, we studied 262 GD patients with long enough follow-up after drug withdrawal to determine treatment outcome. The patients were divided into three groups by time of relapse: early relapse group (n = 91) had an early relapse within 9 months, late relapse group (n = 65) had a relapse between 10 and 36 months, and long-term remission group (n = 106) were either still in remission after at least 3 years or relapsed after 3 years of drug withdrawal. We assessed the treatment outcome of 23 SNPs of costimulatory genes, phenotype and smoking habits. We used permutation to obtain p values for each SNP as an adjustment for multiple testing. Cox proportional hazards models was performed to assess the strength of association between the treatment outcome and clinical and laboratory variables.

**Results:** Four SNPs were significantly associated with disease relapse: rs231775 (OR 1.96, 95% CI 1.18–3.26) at CTLA-4 and rs745307 (OR 7.97, 95% CI 1.01–62.7), rs11569309 (OR 8.09, 95% CI 1.03–63.7), and rs3765457 (OR 2.60, 95% CI 1.08–6.28) at CD40. Combining risk alleles at CTLA-4 and CD40 improved the predictability of relapse. Using 3 years as the cut-off point for multivariate analysis, we found several independent predictors of disease relapse: number of risk alleles (HR 1.30, 95% CI 1.09–1.56), a large goiter size at the end of the treatment (HR 1.30, 95% CI 1.05–1.61), persistent TSH-binding inhibitory Ig (HR 1.64, 95% CI 1.15–2.35), and smoking habit (HR 1.60, 95% CI 1.05–2.42). **Conclusion:** Genetic polymorphism of costimulatory genes, smoking status, persistent goiter, and TSH-binding inhibitory Ig predict disease relapse.

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## Introduction

For patients with Graves' disease (GD), there are three choices of treatment, none of which is perfect. The primary goal of antithyroid drug therapy is to temporarily restore the patient to the euthyroid state while awaiting a spontaneous remission. However, hyperthyroidism re-

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2235-0640/12/0014-0251\$38.00/0  
Accessible online at:  
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curred in 30–60% of GD patients who discontinued the antithyroid medication [1–5]. Understanding of predictors of relapse of disease might help clinicians better individualize their patient's treatment plans. A review of the literature revealed that genetic markers might be used to predict the course of disease after the withdrawal of antithyroid drugs [6, 7]. Goiter size, as a phenotype response to TSH-receptor stimulation, has been reported to be a significant predictor of relapse [1, 8]. Environmentally, iodine intake is a known factor for disease relapse [9, 10]. Smokers are also more likely to experience a relapse than nonsmokers [7, 11].

In this study, we analyzed the factors influencing outcome of antithyroid drug treatment for GD patients in Taiwan, an island where iodine intake is sufficient [12, 13]. Factors studied included both afferent (CTLA-4, CD28, ICOS) and efferent (CD40) costimulatory genes, smoking habits and clinical characteristics including serum levels of  $T_4$ ,  $T_3$ , goiter size, antithyroid medication and treatment duration, and TSH-receptor antibodies at the beginning and end of treatment.

## Methods

### Subjects

From August 2001 until July 2007, 310 GD patients attending the Endocrine Clinic at Kaohsiung Chang Gung Memorial Hospital agreed to the research project. Until December 2010, 262 of the 310 patients (214 women and 48 men aged  $38.1 \pm 12.9$  years) had completed the treatment course and long enough follow-up after drug withdrawal to determine treatment outcome. GD was diagnosed as when a patient had elevated serum thyroxine ( $T_4$ ) and/or triiodothyronine ( $T_3$ ) and suppressed thyroid-stimulating hormone (TSH) levels, diffusely increased thyroidal uptake of technetium-99m or iodine-131, and the presence of TSH-receptor antibodies and/or antimicrosomal antibodies. We excluded patients with a history of radioiodine therapy or previous thyroid surgery. The 200 controls (103 women and 97 men aged  $51.9 \pm 12.2$  years) were recruited from the Health Screening Center of the hospital. They had no clinical evidence or family history of any autoimmune disease. Laboratory tests and physical examination found all controls to be euthyroid state and have no obvious goiter. Informed consent was provided by all patients and controls. This study adheres to the guidelines set forth by the Declaration of Helsinki and was approved by the review board of Chang Gung Memorial Hospital.

### Treatment and Follow-Up

The study group received methimazole 30 mg or propylthiouracil 300 mg daily. Dosage was decreased to two-thirds or half of the initial dose when normal levels of  $T_4$  and  $T_3$  were achieved, usually at 1–2 months after beginning treatment. The dose was then titrated gradually to reach a maintenance dose of methimazole 5–10 mg or propylthiouracil 50–100 mg daily by the third to

fourth month. Low-dose block and replace therapy (methimazole 5–10 mg together with L-thyroxine 50  $\mu$ g) was used in 43 patients to maintain euthyroid levels. Antithyroid drug treatment ranged from 1 to 3 years. Patients who responded to treatment well and reached euthyroid state smoothly usually stopped the medication after 12-month treatment. Patients who had big goiters and/or difficulty to taper the dosage of drugs usually continued the medication for 24 or 36 months. After drug therapy was stopped, patients were asked to return for follow-up studies every 3 months during the first year and every 6 months afterwards. Relapse was defined as a recurrence of symptoms of hyperthyroidism and laboratory data of serum  $T_4$  and/or  $T_3$  exceeding normal range. The 262 patients were divided into three groups according to the time of relapse. Early relapse group ( $n = 91$ ) had an early relapse within 9 months after drug therapy was ceased, those in late relapse group ( $n = 65$ ) had a relapse between 10 and 36 months after drug therapy was ceased, and those in long-term remission group ( $n = 106$ ) were either still in remission after at least 3 years or relapsed after 3 years of drug withdrawal.

### Evaluation of Patients

Clinical and laboratory evaluation included: the genotypes of CTLA-4, CD28, ICOS, and CD40; serum levels of  $T_4$ ,  $T_3$  and TSH; goiter size and TSH-binding inhibitory Ig at the beginning and end of treatment. Goiter size was determined by palpation and classified as: grade 1, a palpable goiter not reaching the medial edge of the sternocleidomastoid muscle; grade 2, a palpable goiter reaching the sternocleidomastoid muscle but not exceeding the lateral edge, and grade 3, a palpable goiter exceeding the lateral edge of the sternocleidomastoid muscle. Serum  $T_4$ ,  $T_3$ , and TSH levels were measured by radioimmunoassay, and TSH-receptor antibody was measured as TBII by radioreceptor assay (TR-AB; CIS Bio International).

Information about the patients' smoking habits was obtained by asking each patient, 'Do you or have you ever smoked daily?' All who answered 'yes' were classified as smokers. Smokers were defined as ex-smokers if they had refrained from smoking for more than 1 year; otherwise, they were considered current smokers. Only current smokers were defined positive for smoking regardless of how much they smoked. 38 of the 39 smokers were male. 24 of the smokers were 40 years old or younger, and 15 were over 40 years old.

### SNP Selection and Genotyping

We examined eight publicly available polymorphisms for CD28, three for CTLA-4, six for ICOS, and six for CD40. The SNPs were selected according to (1) the tagging SNPs from HapMap project (<http://hapmap.ncbi.nlm.nih.gov/>) or (2) the minor allele frequency (MAF)  $\geq 5\%$  in our population based on a pilot sample of 40 GD patients and 40 controls. DNA was extracted from peripheral blood leukocytes. The SNPs of the candidate genes were genotyped by PCR-RFLP for the SNPs at CTLA-4 as previously described [6] or TaqMan technology (Applied Biosystems, Foster City, Calif., USA) for the SNPs at CD28, ICOS and CD40. The position, MAF (based on our control data) and numbers of subjects genotyped are shown in online supplementary data 1 (for all online suppl. material, see online Supplementary Materials). The genotype distributions of all SNPs in the patients and controls are shown in online supplementary data 2.

**Table 1.** SNPs associated with disease relapse

SNPs showing different genotype frequencies among three groups of patients					SNPs predicting disease relapse			
SNP	early relapse	late relapse	long-term remission	p <sup>a</sup>	risk vs. protective genotype	OR	95% CI	p
<i>CD28</i>								
rs1879877								
A/A	47 (51.6%)	18 (27.7%)	35 (33.3%)	0.017	only associated with relapse within 9 months			
A/C	35 (38.5%)	34 (52.3%)	55 (52.4%)					
C/C	9 (9.9%)	13 (20.0%)	15 (14.3%)					
rs3181113								
G/G	21 (23.3%)	18 (28.6%)	26 (24.8%)	0.031	only associated with relapse within 9 months			
G/T	36 (40.0%)	35 (55.6%)	57 (54.3%)					
T/T	33 (36.7%)	10 (15.9%)	22 (21.0%)					
<i>CTLA-4</i>								
rs5742909								
C/C	82 (91.1%)	58 (89.2%)	83 (79.0%)	0.025	only associated with relapse within 3 years			
C/T	7 (7.8%)	7 (10.8%)	22 (21.0%)					
T/T	1 (1.1%)	0	0					
rs231775								
A/A	7 (7.7%)	2 (3.1%)	9 (8.6%)	0.018	*G/G vs. A/G, A/A	1.962	1.182–3.258	0.009
A/G	19 (20.9%)	24 (36.9%)	43 (41.0%)					
G/G	65 (71.4%)	39 (60.0%)	53 (50.5%)					
<i>CD40</i>								
rs745307								
C/C	82 (90.1%)	63 (96.9%)	105 (99.1%)	0.007	*C/T vs. C/C	7.966	1.013–62.651	0.020
C/T	9 (9.9%)	2 (3.1%)	1 (0.9%)					
rs11569309								
C/T	9 (10.2%)	2 (3.4%)	1 (1.0%)	0.009	*C/T vs. T/T	8.088	1.027–63.672	0.019
T/T	79 (89.8%)	57 (96.6%)	100 (99.0%)					
rs3765457								
A/A	72 (79.1%)	56 (91.8%)	97 (93.3%)	0.005	*A/G vs. A/A	2.598	1.075–6.278	0.029
A/G	19 (20.9%)	5 (8.2%)	7 (6.7%)					

Odds ratio (OR) was calculated in SNPs associated with both the 9-month- and 3-year-relapses.

\* Relapse-susceptible genotype.

<sup>a</sup> Test for the genotype difference among three groups of GD by permutation method.  $p < 0.05$  was considered to be significant.

### Statistics

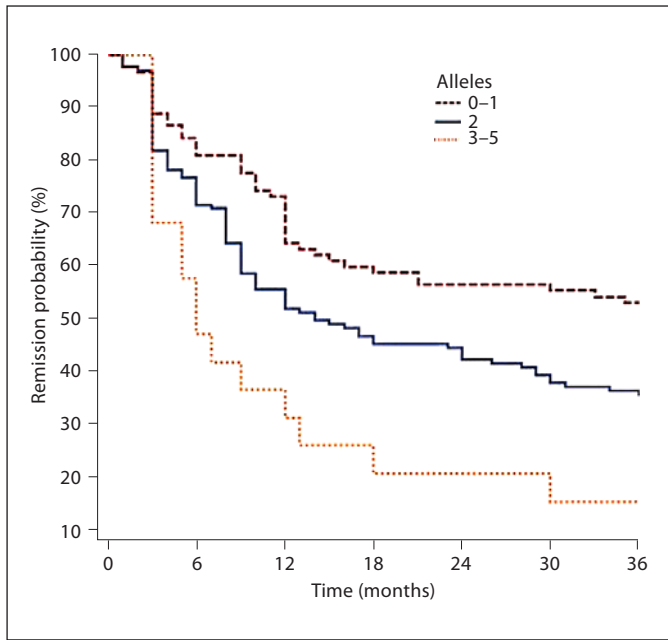
Allele frequencies were estimated by direct gene counting. Hardy-Weinberg equilibrium was tested for each polymorphism by the  $\chi^2$  test. Comparisons of individual clinical and laboratory variables between early relapse, late relapse and long-term remission groups were assessed by one-way ANOVA for the continuous data, and the  $\chi^2$  test or Fisher exact test for the categorical data. Cox proportional hazards models were performed to assess the strength of association between the length of remission and the clinical and laboratory variables. The Kaplan-Meier plot was used to compare patients who remained in remission on MedCalc software. We used permutation to obtain empirical p value for each SNP as an adjustment for multiple testing.

### Results

#### *Genes Associated with Disease Relapse*

Comparison of genotype distributions of all SNPs between patients and controls and among the three subgroups of patients are shown in online supplementary data 2. The genotype distributions were found to be in Hardy-Weinberg equilibrium in the patients and controls for all SNPs, except for rs4335928 at ICOS.

Table 1 specified the 7 SNPs showing difference of genotype frequencies among the three groups of patients. Among these SNPs, rs1879877 and rs3181113 at CD28 were only associated with relapse within 9 months, while



**Fig. 1.** Proportion of patients remaining in remission according to the number of risk alleles, calculated with Kaplan-Meier plot ( $p = 0.0008$ ).

**Table 2.** Distribution of genotypes of CTLA-4 and CD40 in the GD patients

CTLA-4 rs231775 A/G		CD40: rs745307		rs3765457		rs11569309	
		C/C	C/T	A/A	A/G	T/T	C/T
<b>A/A + A/G</b>							
Remission	(n = 52)	52	0	48	2	50	0
Relapse	(n = 52)	48	4	40	11	44	4
<b>G/G</b>							
Remission	(n = 53)	52	1	48	5	50	1
Relapse	(n = 104)	97	7	88	13	92	7

rs5742909 at CTLA-4 was only associated with relapse within 3 years. Therefore, we accounted the significant genes and SNPs associated with disease relapse as CTLA-4 (rs231775) and CD40 (rs745307, rs11569309 and rs3765457), which associated with both the 9-month- and 3-year relapses. Using the cutoff point of 3 years to define relapse and remission, a summary of the odds ratio, confidence interval and p value of the 4 relapse-susceptible genes is shown in table 1.

### Combining Risk Genotypes at CD40 and CTLA-4 Gene Improved Predictability of Relapse

As seen in table 2, a summary of genotype distributions of SNPs at CTLA-4 (afferent costimulatory pathway) and CD40 (efferent costimulatory pathway) in our patients, each patient could carry zero, one, or two risk genotypes from the combination of one risk genotype of CD40 and CTLA-4. When exon 1 +49 A/G at CTLA-4 and rs745307 at CD40 were paired, 8 patients carried two risk genotypes, 153 patients carried one risk genotype, and 100 patients did not carry any risk genotype. The relapse rate was 87.5% for patients with two risk genotypes, 66% for one risk genotype, and 48% for zero risk genotype. The relapse rates among patients with two, one and zero risk genotypes were statistically significant ( $p = 0.005$ ). Similarly, when exon 1 +49 A/G at CTLA-4 and rs3765457 at CD40 were paired, the relapse rates were 72.2% for two risk genotypes, 67.3% for one, and 45.5% for no risk. The relapse rates among patients with two, one and zero risk genotypes were statistically significant ( $p = 0.003$ ). When exon 1 +49 A/G at CTLA-4 and rs11569309 at CD40 was paired, the relapse rates were 87.5% for two risk genotypes, 65.8% for one, and 47.8% for no risk. The relapse rates among patients with two, one and zero risk genotypes were statistically significant ( $p = 0.004$ ).

### Number of Relapse Risk Alleles and Time of Recurrence after Drug Withdrawal

Of the GD patients with known times of relapse in this series, they were divided into three subgroups according to the numbers of risk alleles they harbored: 0–1 risk alleles (90 cases), 2 risk alleles (138 cases), and 3–5 risk alleles (19 cases). The proportion of patients remaining in remission was calculated with Kaplan-Meier plot. We found a significant difference among the subgroups ( $p = 0.0008$ ) (fig. 1).

### Independent Predictors of Disease Recurrence

Univariate analysis of predictors of disease relapse (age, sex, initial serum levels of  $T_4$ , antithyroid drug, treatment duration, presence of ophthalmopathy, goiter size and TSH-receptor antibodies at the beginning and end of treatment, smoking habits, and number of relapse risk alleles) revealed the significant predictors of relapse to be big goiter size at end of treatment ( $p < 0.001$ ), positive TSH-receptor antibodies at the end of treatment ( $p < 0.001$ ), smoking habit ( $p = 0.025$ ), and number of risk alleles ( $p = 0.015$ ), while longer periods of treatment were associated with higher rate of relapse ( $p = 0.001$ ) (table 3).

**Table 3.** Univariate analysis of factors affecting relapse of disease

	Early relapse group	Late relapse group	Long-term remission group	p value
Female/male	78/13	48/17	91/15	0.086
Age, years	37.9 ± 13.6	36.7 ± 13.4	39.1 ± 12.0	0.499
Initial T <sub>4</sub> , µg/dl	18.3 ± 4.3	16.9 ± 4.5	17.0 ± 4.0	0.129
Initial T <sub>3</sub> , µg/dl	318.3 ± 131.9	281.8 ± 94.3	340.8 ± 129.1	0.104
Antithyroid drugs				
Propylthiouracil	27	13	39	
Methimazole	49	36	55	0.084
Block and replace therapy	15	16	12	
Ophthalmopathy				
Positive/negative	15/76	11/54	12/94	0.482
Duration of treatment, months	29.5 ± 22.1	21.4 ± 11.1	21.4 ± 11.7	0.001
Goiter size before treatment				
Grade 0+1	17	20	32	
Grade 2	36	23	44	0.167
Grade 3	38	22	29	
TBII before treatment				
Positive	60	40	58	
Negative	12	5	13	0.575
Goiter size after treatment				
Grade 0+1	27	34	68	
Grade 2	40	18	20	<0.001
Grade 3	22	9	11	
TBII after treatment				
Positive	45	17	19	
Negative	30	36	69	<0.001
Smoking habit				
Positive	13	16	10	
Negative	78	49	96	0.025
Number of relapse-susceptible alleles				
0	3	2	10	
1	17	22	41	
2	58	37	50	0.015
3	5	2	4	p trend <0.001
4	2	1	0	
5	6	1	1	

Early relapse group: relapse within 9 months after drug withdrawal. Late relapse group: relapse between 10 and 36 months after stopping treatment. Long-term remission group: either still in remission after at least 3 years or relapsed after 3 years of drug withdrawal.  $p < 0.05$  is considered statistically significant.

Using the cutoff point of 3 years to define relapse and remission, multivariate analysis revealed that the number of risk alleles, goiter size and TBII level at the end of treatment, and smoking to be independent predictors of relapse. One additional risk allele the patient harbored increased the hazard ratio (HR) by 1.30 (95% CI 1.09–1.56). The HR for each grade of goiter size was 1.30 (95% CI 1.05–1.61), for positive TBII at the end of the treatment 1.64 (95% CI 1.15–2.35), and for smoking habit 1.60 (95% CI 1.05–2.42) (table 4). Further dividing the patients into two subgroups: those below the age of 40 and those above

**Table 4.** Cox proportional hazard models for determinants of risk of relapse

Variable	Adjusted HR	95% CI	p value
Relapse alleles, n	1.30	1.09–1.56	0.003
TBII after treatment	1.64	1.15–2.35	0.006
Goiter size after treatment	1.30	1.05–1.61	0.014
Smoking	1.60	1.05–2.42	0.027



the age of 40, we found the genetic risk alleles (HR 1.24, 95% CI 1.01–1.53) and positive TBII (HR 1.93, 95% CI 1.22–3.06) to be independent predictors in the younger subgroup, but only goiter size at the end of treatment (HR 1.56, 95% CI 1.09–2.22) to be an independent predictor in the older subgroup.

## Discussion

In this study, the genetic factors we chose to analyze were the costimulatory genes, which are not involved in the initiating of autoimmune response to thyroid antigens, but required to generate subsequent cytokines and cell surface molecules after initial binding of T-cell receptor to the antigenic peptide-MHC complex [14, 15]. Because this study focused on the relapse of GD, costimulatory genes play a more crucial role than the genes responsible for disease susceptibility such as HLA or TSHR [16–18]. HLA-DR typing has been proven to be unsuitable for predicting the clinical course of GD patients in a prospective multicenter study [8]. We found associations between 4 SNPs of the costimulatory genes and disease relapse after antithyroid drug withdrawal. In the afferent signal of costimulatory molecules, SNP exon 1 +49A/G (rs231775) of the CTLA-4 gene was found to be a risk allele for the recurrence of GD. In the efferent signal, we found three risk alleles at the CD40 to be associated with relapse. Furthermore, pairing risk alleles at CTLA-4 (afferent signal) and CD40 (efferent signal) improved the predictability of relapse. These findings suggest coordination among afferent and efferent pathways in autoimmune regulation.

In the afferent signal, members of the CD28 gene family (CD28, CTLA-4 and ICOS) are major costimulators located within a stretch of 300 kb on human chromosome 2q33. Linkage of the 2q33 region to thyroid antibodies production has been demonstrated by a genome-wide scan in 56 [19] and 99 families [20] with autoimmune thyroid diseases. The three genes have overlapping and distinct functions in signaling pathways [21]. CD28 is expressed constitutively on CD4 T cells, and its ligand, the B7 family, is present or induced on most activated antigen-presenting cells [22, 23]. In general, naive or primary T-cell responses are more dependent on CD28 than are secondary responses [24]. CTLA-4, which is expressed after T-cell activation, is homologous to CD28 and binds to the B7, which in turn delivers a negative signal to the T cell [25]. CD28 and CTLA-4 represent the ‘general switches’ and strongly influence the expression of many downstream costimulators [15].

In the efferent signal, CD40, on chromosome 20q11.2, is mainly expressed in the B cells and thyrocytes. CD40 interacts with the CD40 ligand (CD40L) on the T cells [26]. CD40L, a member of the TNF family, is the most important mediator of efferent signals that come from the T-helper cells. CD40L is expressed briefly on CD4 T cells after they are activated through the T-cell receptor. CD40 promotes the formation of germinal centers and memory B cells and contributes to B-cell proliferation. CD40L is essential for the crosstalk of T cells with B cells [14, 27]. Based on the above description, it is reasonable to assume that CD40 can affect both the development and progress of GD. In humans, the SNP (rs1883832) C>T at position -1 at the CD40 gene has been linked with GD susceptibility [28–31]. The present study of a Taiwanese population found SNPs at this CD40 for both disease susceptibility (rs3765457, rs4810485 in online suppl. data 2) and disease relapse (rs745307, rs11569309, rs3765457 in table 1).

Kim et al. [32] did not find an association between relapse of GD and the disease-susceptible alleles of CD40 and CTLA-4 genes. Their lack of such a finding may be due to the fact that they divided the GD patients into remission and failure groups with a cutoff point of euthyroid status for 12 months after withdrawal of treatment. Theoretically, an autoimmune disease should be viewed as probable recurring disease. Because of this probable recurrence, length of remission may be a more suitable evaluation method than dichotomizing the patients into success and failure.

This study found smoking to be a strong predictor of relapse of GD. Although smoking has been recognized as a risk for the development of clinically overt thyroid disease [33], increased risk for the development of more severe ophthalmopathy [34, 35] and decreased effectiveness of medical treatment of eye problems [36], few studies have compared response of smoking status to antithyroid drug treatment of GD patients [37, 38]. In one study, using a decline of TSH-receptor antibody and thyroid hormones as a measure, patients with GD who smoked have been reported to respond more slowly to treatment with carbimazole [37]. In another, smoking and male gender were associated with propylthiouracil treatment failure [38]. This study confirms the previous reports of smoking on recurrence after drug withdrawal in Belgian [11] and Taiwanese [7] patients with GD. The prevalence of smoking among Taiwanese men is high (39.0–62.6%), but low among Taiwanese women (2.3–5.3%) [39]. Based on our findings and those of others, patients should be told the importance of quitting smoking before antithyroid drug treatment is initiated.

In our study, clinical parameters of persistent large goiter and high TBII levels at the end of treatment are very useful predictors of disease relapse; a large goiter and a high TBII level at the beginning of treatment, however, were not predictors of disease relapse. Our finding that only persistent high TSH-receptor antibodies titer after therapy predict relapse are supported by previous studies [7, 8, 11, 40] and a meta-analysis of 18 published reports [41]. We found that the relapse rate was higher for longer periods of treatment (table 3). This finding might well be related to our policy of prolongation of antithyroid drug treatment in patients with big goiters and/or difficulties with maintaining euthyroidism with tapering antithyroid drugs. Anyway, our results are in agreement with previous meta-analyses indicating that prolonged treatment with antithyroid drugs beyond 12–24 months does not lower relapse rate. Clinical parameters or phenotype usually reflects the overall interaction between genetic and environmental factors, and are, thus, related to outcome of drug treatment. However, they cannot, as a rule, be assessed at the very beginning of the disease.

In our subgroup analysis by age, genetic factors (risk alleles) were found to be better predictors of recurrence in the younger group, while clinical sign (goiter size) was more useful in the older group. These results are consistent with the genetic studies of type 2 diabetes [42, 43], which have also reported that genetic factors are useful for disease prediction for younger populations. When an individual carrying the risk alleles gets old enough to develop the characteristic phenotypes, the predictive value

of genotype will not override the predictive value of the phenotype.

This study has some limitations. The sample size was small. Because long-term follow-up was needed to clarify the outcomes, we could only include the patients who had been followed up for sufficient time to classify them into the early relapse, late relapse, or long-term remission groups. In addition, we did not analyze all the candidate genes involved in the autoimmune process (e.g. HLA, TSHR, thyroglobulin) [44] and the interaction between the costimulatory genes. These genes and their interaction may have their own impact on outcomes.

## Conclusion

We found genetic polymorphism of costimulatory genes and smoking to contribute to the recurrence of GD, and persistent large goiter and high TBII levels at the end of treatment to be useful predictors of disease relapse (table 4).

## Acknowledgement

This work was supported by a grant (NSC 95-2314-B-182-065) from the National Science Council (ROC).

## Disclosure Statement

The authors have no conflicts of interest to disclose.

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