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The Role of Cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) Gene, Thyroid Stimulating Hormone Receptor (TSHR) Gene and Regulatory T-cells as Risk Factors for Relapse in Patients with Graves Disease

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ABSTRAK

Latar belakang: *penyakit Grave atau* Graves disease *(GD) merupakan kondisi yang umum dijumpai pada tirotoksikosis. Tatalaksana GD diawali dengan pemberian obat antitiroid, meskipun pasien memerlukan waktu lama untuk mencapai kesembuhan atau remisi. Pada kenyataannya, lebih dari 50% pasien yang mengalami remisi masih berisiko mengalami kekambuhan (relaps) setelah obat dihentikan. Penelitian ini bertujuan untuk menilai peran faktor klinis seperti kebiasaan merokok, derajat oftalmopati, derajat pembesaran tiroid, faktor genetik misalnya gen CTLA-4 pada nukleotida 49 di kodon 17 pada ekson 1, gen CTLA-4 pada promotor 318, polimorfisme gen TSHR rs2268458 pada intron 1 dan faktor imunologi sperti sel-sel T regulator (Treg) dan antibodi reseptor tiroid (TRAb) yang memengaruhi terjadinya relaps pada pasien dengan penyakit Grave di Indonesia.* ***Metode:*** *penelitian ini merupakan studi kasus kontrol yang membandingkan 72 subjek dengan relaps dan 72 subjek tanpa relaps pada 12 bulan setelah penghentian pengobatan antitiroid, yang memenuhi kriteria inklusi. Pemeriksaan polimorfisme genetik dilakukan menggunakan PCR-RFLP. Jumlah sel T regulator dihitung menggunakan analisis sitometri alir (flow cytometry) dan pemeriksaan ELISA untuk mengukur TRAb. Regresi logistik dilakukan karena variabel dependen adalah variabel kategorik.* ***Hasil:*** *terdapat korelasi antara kekambuhan penyakit dan faktor keluarga (p=0,008), usia saat diagnosis ditegakkan (p=0,021), oftalmopati Graves derajat dua (p=0,001), pembesaran kelenjar tiroid yang melebihi batas lateral otot sternokleidomastoideus (p=0,040), lamanya masa remisi (p=0,029), genotipe GG dari gen CTLA-4 pada nukleotida 49 di kodon 17 dari ekson 1 (p=0,016), genotipe CC dari gen TSHR pada rs2268458 dari intron 1 (p=0,003), jumlah sel T regulator (p=0,001) dan kadar TRAb (p=0,002).* ***Kesimpulan:*** *polimorfisme genetik gen CTLA-4 pada nukleotida 49 di kodon 17 pada ekson 1, gen TSHR SNP rs2268458 pada intron 1, jumlah sel T regulator dan kadar TRAb berperan sebagai faktor risiko terjadinya relaps pada pasien penyakit Graves.*

Kata kunci: *penyakit Grave, relaps (kekambuhan), gen CTLA-4 ekson 1, gen CTLA-4 dengan promoter -318, gen TSHR rs2268458 intron 1, sel-sel T regulator, Antibodi Reseptor Tiroid (TRAb).*

ABSTRACT

Background: *graves' disease (GD) is the most common condition of thyrotoxicosis. The management of GD is initiated with the administration of antithyroid drugs; however, it requires a long time to achieve remission. In reality more than 50% of patients who had remission may be at risk for relapse after the drug is stopped. This study aimed to evaluate the role of clinical factors such as smoking habit, degree of ophtalmopathy, degree of thyroid enlargement; genetic factors such as CTLA-4 gene on nucleotide 49 at codon 17 of exon 1, CTLA-4 gene of promotor -318, TSHR gene polymorphism rs2268458 of intron 1; and immunological factors such as regulatory T cells (Treg) and thyroid receptor antibody (TRAb); that affecting the relapse of patients with Graves' disease in Indonesia.* ***Methods:*** *this was a case-control study, that compared 72 subjects who had relapse and 72 subjects without relapse at 12 months after cessation of antithyroid treatment, who met the inclusion criteria. Genetic polymorphism examination was performed using PCR-RFLP. The number of regulatory T cells was counted using flow cytometry analysis and ELISA was used to measure TRAb. The logistic regression was used since the dependent variables were categorical variables.* ***Results:*** *the analysis of this study demonstrated that there was a correlation between relapse of disease and family factors (p=0.008), age at diagnosis (p=0.021), 2nd degree of Graves' ophthalmopathy (p=0.001), enlarged thyroid gland, which exceeded the lateral edge of the sternocleidomastoid muscles (p=0.040), duration of remission period (p=0.029), GG genotype of CTLA-4 gene on the nucleotide 49 at codon 17 of exon 1 (p=0.016), CC genotype of TSHR gene on the rs2268458 of intron 1 (p=0.003), the number of regulatory T cells (p=0.001) and TRAb levels (p=0.002).* ***Conclusion:*** *genetic polymorphisms of CTLA-4 gene on the nucleotide 49 at codon 17 of exon 1, TSHR gene SNP rs2268458 of intron 1, number of regulatory T cells and TRAb levels play a role as risk factors for relapse in patients with Graves' disease.*

Keywords: *graves' disease, relapse, CTLA-4 gene exon 1, CTLA-4 gene with promotor -318, TSHR gene rs2268458 intron 1, Regulatory T cells, Thyroid Receptor Antibody (TRAb).*

INTRODUCTION

Graves' disease (GD) is the most common condition found in thyrotoxicosis, caused by a complex relationship between genetic factors and environmental influences. The prevalence of GD in America and Europe is about 0.5-1%, while in Indonesia is estimated at about 0.05%.[1-4] Genetic factors that have a role in this condition involve the interaction between Major Histocompatibility Complex (MHC) and non-MHC genes, i.e. imunoregulator genes and specific autoantigen genes. Imunoregulator genes that affect GD development are the cytotoxic-T lymphocyte-associated protein 4 (CTLA-4) gene, CD40 gene and protein- tyrosine phosphatase-22 (PTPN22) gene; while the thyroid-specific genes are thyroglobulin and thyroid stimulating hormone receptor (TSHR) gene.[5-8]

Graves' disease is an autoimmune disease of the thyroid gland caused by impaired balance of T helper cell 1 (Th1) and T helper 2 (Th2). The role of Th2 cells is more profound, but at the initial stage of GD, the role of Th1 cells is predominant.[9-11] Recent studies also demonstrated the role of regulatory T cells (Treg), which are also derived from naive Th cell and controlling the activity of T cells through the co-stimulation process of dendritic cells.[12-16]

The management of GD is aimed to treat symptoms caused by hyperthyroidism. The goal of treatment is to control over-production of thyroid hormone and reducing the enlargement of thyroid gland. It includes antithyroid agents, radioactive iodine or surgical treatment such as thyroidectomy. Antithyroid agents are considered as the first-line treatment since it is easily implemented.[17-18] The administration of antithyroid agents requires considerable time to achieve remission, which is at least 12-18 months. In reality, more than 50 percent of patients with GD who had remission may experience repeated symptoms of hyperthyroidism or may have

a relapse. Factors affecting the occurrence of relapse are clinical factors such as gender, age, smoking habit, degree of ophtalmopathy, degree of thyroid enlargement, duration of antithyroid treatment; as well as the genetic factors such as CTLA-4 gene, TSHR gene, thyroglobulin gene; and the immunological factors such as regulatory T cells (Treg) and thyroid receptor antibody (TRAb). This study aimed to evaluate the role of clinical, genetic and immunological factors affecting the relapse of patients with Graves' disease in Indonesia.

METHODS

This was a case-control study, which compared GD patients who had relapsed and those without relapse at 12 months after cessation of antithyroid treatment. Subjects were recruited at outpatient clinic of Metabolism and Endocrinology division, Department of Internal Medicine, Cipto Mangunkusumo Hospital between August and December 2014. As much as 72 subjects who experienced relapse and 72 subjects without relapse, who met the inclusion criteria were obtained. Patients were said to relapse when after the drug was stopped there was an increase in T4 and/or T3 levels accompanied by a decrease in TSH. Definition of the occurrence time of relapse cannot be determined, but generally some researchers agree to use 6 months time periods after the drug was stopped. This study has been approved by Research Ethical Committee of the Faculty of Medicine, Universitas Indonesia (No. 540/UN2. F1/ETHICS/2014).

Parameters evaluated in this research were factors affecting relapse in Graves' disease including clinical factor, genetic factor and immunological factor. Clinical factors included age, gender, age at diagnosis, smoking habit, family history of thyroid disease, degree of ophtalmopathy, degree of thyroid enlargement, duration of antithyroid treatment, duration of euthyroid period after the cessation of antithyroid treatment; genetic factor included CTLA-4 gene on nucleotide 49 at codon 17 of exon 1, CTLA-4 gene of promotor -318, TSHR gene polymorphism rs2268458 of intron 1; and immunological factors included regulatory T cells (Treg) and thyroid receptor antibody (TRAb).

Graves ophtalmopathy (GO) is an inflammatory disorder of the orbit that occurs in association with autoimmune thyroid disease. The degree of severity can be graded using NOSPECS that ranges from 0 to 6. Grade 0 means no sign or symptoms, grade 1 means only signs limited to upper lid retraction and stare with or without lid lag, grade 2 means soft tissue involvement (oedema of conjunctivae and lids, conjunctival injection, etc), grade 3 means proptosis, grade 4 means extraocular muscle involvement and diplopia, grade 5 means corneal involvement, and grade 6 sight loss due to optic nerve involvement.[4] This research used NOSPECS criteria of GO which was further into 3 groups: degree 0 for GO grade 0.1 and 2; degree 2 for GO grade 3 and 4; and degree 3 for GO grade 5 and 6. The degree of enlarged thyroid gland in our study was determined by the criteria used by Wang.[23] The degree of enlargement can be graded from 1 to 3. Grade 1 if the thyroid gland does not reach the medial edge of the sternokleidomastoideus muscle, grade 2 if the thyroid gland reaches the sternocleidomastoideus muscle but not beyond the lateral edge and grade 3 if the thyroid gland exceeds the lateral edge of the sternocleidomastoideus muscle.[23]

Examination of Genetic Polymorphisms

The examination utilized 3 ml EDTA-treated blood for leukocyte isolation using Ficoll-Hypaque centrifugation. Genetic polymorphism examination conducted by DNA isolation using Promega Wizard DNA Purification Kit (Ref A1125) and DNA isolation procedure following the instructions in the kit. Concentration and DNA purity was calculated using nanodrop (Maestro) with a purity range of 1.8-2.0. Amplification of target DNA fragments was one using Polymerase Chain Reaction (PCR). Solution PCR consists of 0.2 μg genomic DNA, 0.5 μL Taq polymerase, 0.5μM primary forward, 0.5μM primary reverse and 200 μM dNTP. The DNA samples were amplified 35 cycles with initial denaturation at 94°C during temperature 5 minutes, then went into the cycle consisted of annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds and over denaturation at 94°C for 30 seconds,

and last extension at 72°C for 7 minutes. After the amplification process, 5 μL of 25 μL PCR reaction was restricted for 3 hours at 37°C. Result of amplification separated by electrophoresis on agarose gel 2.5% for 45 minutes at 90 volts. Visualization of the band of DNA fragments of electrophoresis results was observed with a UV illuminator (UV long lifeTM filter Spectroline). Polimorfism of gene was done with Restriction Fragment Length Polymorphism (RFLP) method, and used restriction endonuclease enzyme.

Polymorphism of gene that were thought to influence relapse in GD and evaluated in this research were CTLA-4 gene on nucleotide 49 at codon 17 of exon 1, CTLA-4 gene of promotor -318 and TSHR gene rs2268458 of intron 1. Examination of genetic polymorphism for CTLA-4 gene on nucleotide 49 at codon 17 of exon 1 was done using Fnu4HI enzyme that cut the DNA sequences into several fragments. We used the 5'-GGCTTGCCTTGGATTTCAACGGC-3' as forward primer and the 5'- GCTTCC AAAAAGTCTCACTCACC-3' as reverse primer were used. Nitrogen base of Guanin (G) when located on position 49 will produce fragmen 98/41 bp, but when Adenin (A) is located on position 49, it does not cause restriction in fragmen 129 bp.

Examination of genetic polymorphism for CTLA-4 gene of promotor -318 was performed by using an MseI restriction enzyme that cut DNA sequences into several fragments. The 5'-AAATGAATTGGACTGGATGGT-3' was used as forward primer and 5'-TTACGAGAAA GGAAGCCGTG-3' was used reverse primer, which cut the DNA sequence into several fragments. Nitrogen base of Timin (T) when located on position -318 produce fragmen 132/115 bp, but when Sitosin (C) is located on position -318, it does not cause restriction in fragmen 247 bp.

Examination of genetic polymorphism for TSHR gene rs2268458 of intron 1 was performed by using restriction enzyme, AluI restriction endonuclease, which cut the DNA sequences into several fragments. The forward primers were 5'-CCAGCAGAGGGAGCACAA-3' and the reverse primers were 5'-TAGAGAATAGAGC AGCAAGACACT-3'. Nitrogen base of Sitosin (C) when located on position intron 1 cut on 275 bp, but when Timin (T) is located on intron 1, it cut on 333 bp.

Examination of Regulatory T cells (Treg)

Examination of Treg cells was performed using Human Regulatory T Cell Cocktail BD PharMingen Kit. The surface sections were stained with CD4FITC (fluoresceinsothiocyanate) and 10 μL CD25-PE (R-phycoerythrin); while the intracellular portion was stained with antibody anti-FoxP3-PE (R-phycoerythrin). The number of regulatory T cells was calculated with the BD FACS flowcytometer (CaliburTM®).

Thyroid Receptor Antibody (TRAb) Serum Levels

TRAb serum levels were measured by ELISA method using R & D system kits.

Statistical Analysis

Data were analyzed using SPSS version 20.0. In bivariate analysis, the numeric variables were evaluated using T-test when there was a normal distribution and Mann Whitney or Kruskal-Wallis test were used when the data were not normally distributed. The bivariate analysis for categorical variables used Chi-square test, Fischer or Kolmogorov Smirnov test. For the multivariate analysis, the logistic regression was used since the dependent variables were also categorical variables.

RESULTS

The results of the present study indicated that the age at diagnosis for patients with GD in this study was mostly less than 30 years old (p=0.036) and the patients who also had family member suffering from the disease would have a higher risk for relapse (p=0.008). Graves' ophthalmopathy with corneal involvement or sight loss (the 2nd degree of ophthalmopathy), enlarged thyroid gland exceeding the lateral edge of sternocleidomastoid muscle (the 3rd degree), and less than 2 years remission period after the cessation of treatment, had greater risk for relapse.

Grave's Disease patients in this study mostly had G allele on gene CTLA-4 nucleotide 49 codon 17 exon 1 compared to A allele (53.5%), C allele on gene CTLA-4 promotor -318 (85.4%) than T allele, C allele on gene thyroglobulin

Table 1. Subjects' characteristics

Variables	Values
Gender: Female, n (%)	120/144 (83.3)
Age, Median (min-max), year	45 (27-77)
Age at diagnosis, median (min-max), year	32 (18-60)
Smoking habit, n (%)	4/144 (2.8)
Family factor, n (%)	24/144 (16.7)
Ophtalmopathy: degree 1 and 2, n (%)	92/144 (63.9)
Thyroid enlargement, degree 2 and 3, n (%)	84/144 (54.2)
Antithyroid drug: PTU, n (%)	80/144 (58.3)
Duration of antithyroid drug treatment, median (min-max) year	2 (1-6)
Lenth of euthyroid period, median (min-max) year	2 (0.5-3.1)
Gene CTLA-4 nucleotide 49 exon 1: alel G, n (%)	154/288 (53.5)
Gene CTLA-4 promotor -318: alel C, n (%)	246/288 (85.4)
Gene TSHRSNP rs2268458 intron 1: alel T, n (%)	170/288 (59.1)

nucleotide 5995 codon 1980 exon 33 (52.1%) than T allele, and T allele on gene TSHR SNP rs2268458 intron 1 (59.1%) than C allele.

Data on serum level of the immune response in the subjects were not normally distributed, thus the median, minimum and maximum values were reported.

In this study, it was obvious that GD subjects with GG genotype of CTLA-4 gene at nucleotide 49 codon 17 of exon 1 had a risk for relapse of 7.3 times higher than those AA genotype; while those with GA genotype of CTLA-4 gene at nucleotide 49 codon 17 of exon 1 had a risk for relapse of 1.4 times higher than those with AA genotype. Graves' Disease subjects with CC genotype TSHR gene SNP rs2268458 of intron 1 were at risk for relapse of 13.3 times higher than those with TT genotype.There was no difference in the risk for relapse between GD subjects with CC and TT genotypes of CTLA-4 gene of promotor -318.

The present study demonstrated that number of Treg cells in GD patients who had relapse was lower (4.43) than those who did not have relapse (9.25). Moreover, the TRAb serum level in the relapse patients (9.65 unit/L) were higher than those who did not have relapse (4.36 u/L). Statistical analysis showed a significant correlation between the risk of relapse with the number of Treg cells (p 0.001) and TRAb serum level (p 0.002).

Table 2. Characteristics of immune response

Variables	Median (min - max)
Number of regulator T cells	6.85 (0.89 - 24.36)
Level of TRAB (unit/L)	5.45 (0.3 - 25.95)

DISCUSSION

Most subjects in this study were female (83.3%). This finding is consistent with the the epidemiological data that the prevalence of Graves' disease is mostly female with a ratio of 5: 1 to male.[1,2] The role of age on the risk for relapse can be evaluated from the presence of age differences at the time of GD diagnosis. within in the group who experienced relapse, the mean age of patients at the time of first diagnosis was 28.38 years and in the group without relapse, was 36.12 years (p=0.021). It indicates that the younger the age of GD patients, the risk of relapse was higher. A study conducted by Stefanie,[19] which compared the onset of GD development between two groups, also found similar results.

Tobacco in cigarettes has the inflammatory effect that can lead to thyroid gland damage and immunomodulatory effects since it can suppress the activation of T cells, reduce the performance of T-killer cells and may cause defects in humoral and cellular immunity. Smoking can also decrease the effectiveness of antithyroid treatment and increase the growth of thyroid gland.[20] These factors cause GD patients who are still actively smoking to have difficulty in remission and greater risk for opthalmopathy.[21] The present study demonstrated that although statistically there was no risk difference of relapse between the smoker and the non-smoker subject (p=0.31), all of GD patients with smoking habits experienced relapse. The remission period in smoker GD patients only last for 8-10 months and none of the non-smoker subjects had experienced relapse.

Our study also provided evidences that GD patients who had family member with GD actually had risk for relapse of 2.137 times greater compared to those without family history of GD.

Table 3. Risk for relapse in the subjects based on clinical factors

	Relapse	No relapse	P*	OR (95% CI)
Sex, n (%)				
- Female	64 (88.9)	56 (77.8)	0.439*	0.714 (0.278-1.832)
- Male	8 (11.1)	16 (22.2)		
Age at diagnosis, n (%)				
- <30 years	40 (55.6)	14 (19.4)	0.036*	1.970 (1.136-3.416)
- ≥ 30 years	32 (44.4)	58 (80.6)		
Smoking habit, n (%)				
- Smoking	4 (5.6)	0 (0)	0.312**	0.798 (0.238-1.053)
- No smoking	68 (93.4)	72 (100.0)		
Family factor, n (%)				
- Positive	42 (58.3)	14 (19.4)	0.008*	2.14 (1.210-3.775)
- Negative	30 (41.7)	58 (80.6)		
Degree of ophtalmopathy				
- 2nd degree	6 (8.3)	2 (2.8)	0.011***	6.50 (0.460-11.924)
- 1st degree	54 (75.0)	30 (41.6)	0.166***	5.85 (1.499-9.825)
- 0 degree	12 (16.7)	40 (55.6)		
Degree of thyroid enlargement				
- 3rd degree	16 (22.2)	4 (5.6)	0.040***	11.67 (1.112-22.381)
- 2nd degree	38 (52.8)	26 (36.1)	0.063***	3.370 (0.938-11.115)
- 1st degree	18 (25.0)	42 (58.3)		
Antithyroid drug				
- PTU	50 (69.4)	30 (41.7)	0.38*	1.667 (0.528-5.265)
- Non PTU	22 (30.6)	42 (58.3)		
The duration of treatment using antithyroid drug				
- < 2 years	16 (22.2)	18 (25.0)	0.731*	0.789 (0.205-3.047)
- ≥ 2 years	56 (77.8)	54 (75.0)		
The length of euthyroid period after the cessation of antithyroid treatment				
- < 12 months	32 (44.4)	12 (16.7)	0.029*	1.862 (1.105-3.137)
- ≥ 12 months	40 (55.6)	60 (83.3)		

* chi-square test; ** Fisher test; *** Kolmogorov Smirnov

Table 4. Risk for relapse based on CTLA-4 gene at nucleotide 49 codon 17 of exon 1

CTLA-4 exon 1	Relapse	No relapse	P*	OR (95% CI)
GG Genotype	38 (52.8)	12 (16.7)	0.016	7.312 (1.439-37.164)
GA Genotype	22 (30.5)	32 (44.4)	0.642	1.432 (0.321-6.492)
AA Genotype	12 (16.7)	28 (38.9)		

* Chi-square test

Table 5. Risk for relapse based on gene CTLA-4 gene of promotor -318

CTLA-4 promoter	Relapse	No relapse	P*	OR (95% CI)
CC Genotype	60 (83.3)	42 (58.3)	0.104	1.857 (0.782-4.408)
CT Genotype	12 (16.7)	30 (41.7)		
TT Genotype	0 (0.0)	0 (0.0)		

* Chi-square test

Table 6. Risk for relapse in the subjects based on immune response

	Relapse (n=72)	No relapse (n=72)	P*
Regulatory T cell	4.43 (0.89-11.3)	9.25 (2.61-24.36)	0.001
TRAb level (unit/l)	9.65 (6.30-25.95)	4.36 (0.33-11.4)	0.002

*Mann-Whitney test

Ophtalmophathy is a clinical marker most frequently found in patients with GD. This study, used the NOSPEC classification for determining ophtalmopathy degree. Patients with 2nd degree ophthalmopathy based on NOSPEC classification, had 6.5 times greater risk for relapse than those of 0 degree. Graves' disease patients with 1st degree ophthalmopathy, who had eyelid retraction and bulging eyes, had 5.85 times greater risk compared to those of 0 degree (p=0.011). It indicates that the higher the degree of ophthalmopathy, that higher risk for relapse is.

Enlarged thyroid gland in patients with GD can be determined by physical examination or by measuring the volume of the thyroid gland using ultrasound. In our study, we did not perform thyroid ultrasound; however we did perform the inspection and palpation of physical examination. The degree of thyroid enlargment can be determined by the criteria used in Wang's study.[23] Based on this classification, the GD patients with the 3rd degree had 11.7 times greater risk for relapse compared to those of 1st degree (p=0.040). Patients with 2nd degree thyroid enlargment, had 3.37 times greater risk for relapse compared to those of 1st degree (p=0.063).

The available antithyroid drugs in Indonesia are PTU and methimazole. PTU is a short-acting drugs that should be given three times a day. Our study demonstrated that the types of antithyroid drugs, whether PTU or methimazole, did not affect the occurrence of relapse, with the significance level of p=0.383. This is also consistent with studies reported by Jonas[24] and Wang[25].

Administration of antithyroid drugs is given for at least 18 months; although, there are some who require longer treatment time.[1,2] In our study, we limited the period of antithyroid treatment for as long as 2 years to determine whether the patients are included in the group of GD with difficult remission or not. The results of our study showed that there was no difference regarding the risk for relapse, between groups of GD patients who received treatment for 2 years compared to those receiving treatment less than 2 years (p=0.731). It demonstrated that the length of therapy does not affect the development of relapse.

The definitions of remission in GD are various in several studies. Allahabadia[26] defines remission as the condition in which the patient remains in a euthyroid state without antithyroid treatment for more than 6 months after the antithyroid treatment was ceased. Our study showed that patients who experienced euthyroid GD less than 12 months after the drug was stopped, had 1.86 times greater risk for relapse than those who continually experience euthyroid for more than 12 months after the drug was stopped. Mohlin[27] reported that when GD patient remained in remission for more than 4 years after the treatment is stopped, the risk for relapse was getting smaller (p=0.001).

The Role of Genetic Polymorphism as the Risk Factor for Relapse

The CTLA-4 gene consists of 4 exons and 3 introns. Exon is the most important part of a gene for carrying the message in the DNA that will be transcripted into mRNA and then translated in the ribosome to be CTLA-4 protein. Exon 1 of CTLA-4 gene has long been known to have a role in various autoimmune diseases. The results of this study demonstrate that polymorphism of CTLA-4 gene of nucleotide 49 codon 17 of exon 1 had not only a role in the risk factor for the development of autoimmune disease, but also as the risk factor for relapse. GD subjects with the GG genotype had 7.31 times greater risk for relapse compared to those with AA genotype; while those with GA genotype had 1.43 times greater risk for relapse compared to subjects with AA genotype. Wang et al[28] also reported that GD subjects with GG genotype would experience the relapse faster (59.6%) compared to those with AA genotype (7.2%) with p=0.0006. Sahin[29] study also reported that GD subjects with GG genotype in Turkey had 14.75 times higher risk for relapse than those with AA genotype.

The structure of gene is composed of a promotor, the structural part and the terminator. Promoter consists of specific DNA sequences located at the front of the structure of gene. The function of promoter is to control gene transcription. Polymorphisms in the promoter of gene may affect the CTLA-4 level by changing the binding of transcription factors. The results of our study found that GD subjects with CC genotype of CTLA-4 gene of promotor -318 had 1.86 times greater risk for relapse compared to those with CT genotype, although it was not statistically significant (p=0.104). Kim[30] failed to demonstrate the role of promotor of CTLA-4 gene as a risk factor for relapse (p=0.109). In contrast, Wang[28] had suggested that there was a role of CC genotype with a significant difference (p=0.0173).

In human, TSHR gene has 191 kb of DNA and is located on the long arm of chromosome 14 (14q31). TSHR gene in intron 1 acts as a regulator for RNA, which affects the location of initial formation of mRNA.[31] A study conducted by Dechairo[32] has demonstrated that the TSHR gene rs2268458 in intron 1 had a role in GD events. A study report by Yin[33] also suggested that the CC genotype of TSHR gene SNP rs2268458 in intron 1 had greater role in GD compared to its role with Hashimoto's thyroiditis patients and healthy subjects. Our study found that the GD subjects with CC genotype for TSHR gene SNP rs2268458 of intron 1 had 13.3 times greater risk for relapse compared to those with TT genotype; moreover, GD subjects with CT genotype had 8.11 times greater risk for relapse compared to those with TT genotype.

The Role of the Immune Response as the Risk Factor for Relapse

Naive Th cells which develop into several subsets of T cells are Th1 cells, Th2 cells, regulatory T cells and Th17 cells. In GD, all of those subsets of T cells have a quite essential role. Regulatory T cells (Treg) with markers of CD4 + CD25 + Foxp3 are able to suppress the innate immune response and the acquired immune response; therefore, when there is a deficiency of Treg cells, there would be an increased activity of autoimmune diseases including GD.[33-38]

Our study demonstrated that the number of Treg cells in GD patients who had experienced relapse was lower than those who did not have experienced relapse. It also showed a statistically significant correlation between the number of Treg cells and the risk for relapse (p=0.001). Pan[34] had measured regulatory T cells using flow cytometry and real-time quantitative RNA analysis in GD patients and healthy subjects and they found that the number of regulatory T cells in patients with GD (10.87) unit was lower than the healthy subjects (13.4) unit.

In patients with GD, thyroid stimulating antibody (TSAb) binds to the TSH receptor, which will activate adenyl cyclase and phospholipase A2 on the receptors. It may lead to the production of thyroid hormones and the growth of thyroid cells (thyrocytes).[39,40] Our study showed a higher level of TRAb in GD patients who had experienced relapse (9.65 U/L) than those who did not have relapse (4.36 U/L); moreover, the statistical analysis also showed a significant correlation with p=0.002. Nedrebo[41] who conducted a prospective study over 12 months periods also demonstrated that the GD patients who had high TRAb level (26 U/L) at the end of treatment period had 3.14 times greater risk for relapse than those with TRAb level of 15.8 U/L.

The results of multivariate analysis with logistic regression showed that the clinical factors that affect the development of relapse in patients with GD is the family history, which showed 4.129 times greater risk for relapse when the patient has a family member who also has suffered from GD and 2nd degree ophthalmopathy which showed 6.342 times greater risk for relapse. The genetic factors that influenced of relapse in GD were GG genotype of CTLA-4 gene at nucleotide 49 codon 17 of exon 1 with a risk of 5.963 times; and those with CC genotype of TSHR gene SNP rs 2268458 of intron 1 with a risk of 7.931 times. Factors associated with immune response that may affect the development of relapse were the low number of regulatory T cells with a risk of 1.587 times and the high TRAb level with a risk of 1.277 times. This research did not analyse the factors with most influence towards relapse in GD, among the clinical factors, genetic factors or immunological factors.

CONCLUSION

The genetic factors that affect the development of relapse in GD are GG genotype of CTLA-4 gene at nucleotide 49 codon 17 of exon 1 and CC genotype of TSHR gene SNP rs2268458 of intron 1. Factors associated with immune response are decrease amount of T regulatory cells and increase level of TRAb serum. The clinical factors determine the recurrence are family history of having GD, edema or injections on conjunctiva or palpebral (the 2nd degree of ophthalmopathy according to the NOSPECS classification), enlarged thyroid gland exceeding the lateral edge of sternocleidomastoid muscle (the 3rd degree) and the length of euthyroid period of less than 6 months and the age at diagnosis for GD of less than 30 years. Patients with those characteristics should not be given prolong antithyroid treatment, but immediately given definitive therapy i.e. radioactive iodine or thyroidectomy.

Further research needs to be conducted to determine the risk of recurrence in GD models, using statistical methods with more number of sample cases and validated prospectively.

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