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POLYMORPHISMS OF PROMOTER GENE FORKHEAD BOX PROTEIN-3 (FOXP3) T-REGULATOR IN PATIENTS WITH GRAVES' DISEASE IN WEST SUMATRA, INDONESIA

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POLYMORPHISMS OF PROMOTER GENE FORKHEAD BOX PROTEIN-3 (FOXP3) T-REGULATOR IN PATIENTS WITH GRAVES' DISEASE IN WEST SUMATRA, INDONESIA

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Background
Forkhead Box Protein 3 (FOXP3) is a gene that controls the development and function of T-regulatory (Treg) cells. Dysfunction of Treg cells can trigger an autoimmune condition caused by a change in FOXP3 promoter gene activity. Aim of this study is to correlate between FOXP3 T-regulator promoter gene polymorphisms in patients with Graves' disease in West Sumatera, Indonesia.

Method
This study was an observational study with cross sectional comparative study design. Consecutive sampling was conducted in patients with Graves' disease who came to outpatient clinic and treated in Dr. M. Djamil Hospital, Padang. Blood sampling was performed on 30 Graves' subjects and 30 healthy control subjects based on inclusion and exclusion criteria. DNA isolation, primary construction, polymorphism identification were analysed by PCR method.

Results
Results of this study obtained the most age of patients with Graves' disease is 30-40 years with female gender. Graves' patient group was found to have SNP rs2232365, SNP rs3761547, SNP rs3761548 and SNP rs3761549 with mutant heterozygote polymorphisms were mostly found, and there was no polymorphism of SNP rs2232364 found in patients with Graves' disease on this study. We also found deletion in SNP FOXP3 which haven't been noted in NCBI which located in base 49, as seen in Figure 3.There was no correlation between polymorphisms of promotor gene FOXP3 in Graves' disease group and healthy control group.

Conclusion
This study proves that there are polymorphisms of promoter gene FOXP3 in patients with Graves' disease, however there is no correlation between polymorphisms FOXP3 promoter gene in Graves' disease compare to control.

Keywords: Promotor Gene, FOXP3, T-regulatory cells, Graves' disease

INTRODUCTION

Graves' disease (GD) is an autoimmune thyroid disease caused by overexpressed autoantibodies resulting in hyperthyroidism, goiter, ophtalmopathy and or dermatopathy [1]. Graves' disease thought to be a T-helper 2 (Th2)-cell driven mediated disease that stimulates production of autoantibodies [2]. Dysfunction of regulatory T cells (Tregs) in maintaining balance between T-helper 1 (Th1) and T-helper 2 (Th2) is one of the factors that may cause the occurrence of autoimmune, especially Graves' disease [3].

Regulatory T cells have been recognized as CD4 T cells that plays a role in maintaining balance between peripheral tolerance and preventing autoimmune disease [4]. One of the best characterized marker of Tregs is the forkhead box protein-3 (FOXP3) which the only one marker that located in intracellular of Tregs cells. FOXP3 gene located on position Xp11.23 in the short arm of X chromosome, consist of promoter region and conserved non-coding DNA sequence (CNS1-3) [5]. Promoter region of FOXP3 is responsible in maintaining differentiation of regulatory T cells in peripheral. Functional mutations in FOXP3 gene promoter have been associated with various autoimmune disease such as psoriasis, SLE, and rhinitis allergy [6]. There are five variants in the promotor region FOXP3 (-924A/G (rs2232365), - 1383C/T (rs2232364), -2383C/T (rs3761549), - 3279C/A (rs3761548) and - 3499A/G (rs3761547)) have been associated with altered function of FOXP3 which may lead to Tregs dysfunction [7].

Aim of this study was to identify the role of FOXP3 gene promoter in association with susceptibility to GD in West Sumatra, Indonesia. This is the first study in population of Indonesia, especially West Sumatra showing association between role of FOXP3 gene promoter and Graves' disease.

METHODS

This was a case-control study, which compared patients diagnosed as Graves' disease based on clinical and laboratory examination and healthy subjects as control. Subjects were recruited at outpatient clinic of Metabolism and Endocrinology division, Department of Internal Medicine, M. Djamil Hospital, Padang, West Sumatra, Indonesia.

As much as 30 subjects with Graves' disease and 30 healthy subjects were recruited as subjects of this study. Diagnosis of GD were based on clinical symptom and occurrence of autoantibody thyroid, TRAb. This study has been approved by Research Ethical Committee of Faculty of Medicine, Andalas University (No.316/KEP/FK/2017).

Age, gender, thyroid antibody (TRAb) were noted as characteristic data of this study. New venous blood sample were taken for measurement of serum FT4, TSH, TRAb and gene polymorphisms. TRAb were measured using a receptor assay, Human TRAb Elisa kit 96T, Bioassay Tech. Lab (TRAb $\geq$ 1,0 IU/L being considered as TRAb positive).

Genomic DNA was extracted from EDTA-anticoagulated blood with standard phenol-chloroform method. Five SNPs of gene promoter FOXP3, -924A/G (rs2232365), - 1383C/T (rs2232364), - 2383C/T (rs3761549), - 3279C/A (rs3761548) and - 3499A/G (rs3761547), was performed using PCR direct DNA method. Primer sequences used were: FOXP3-F1 (5'-ATGCATGTGTCCATTTCTCTC-3'); FOXP3-R1 (5'- TTTCATATCGGGGTCTGGCATC-3'); FOXP3-F2 (5'-CCTGGATTCTCACACTCATCTC-3') and FOXP3-R2 (5'-GAAGGACCGAGCTGACATTAC-3').

Data were analyzed using SPSS version 20.0. The distribution of genotypes in both groups was compared using the chi-squared test. Data were expressed as mean $\pm$ SD with level of significance set at $p<0,05$.

RESULTS

Baseline characteristic of patients and control

Thirty GD patients and healthy control subjects were enrolled in this study. Most GD patients were female (76,7%), therefore to maintain uniformity 76,7% healthy control were also female. Mean of age of this study were 40,23 ± 9,98 years old, with age between 31-40 was the most common age found in this study. As shown in table 1, the levels of TRAb were significantly higher in GD patients compared to control.

Table 1. Baseline Characteristics of Graves' Patients and Control

Characteristic	Graves' patients		Control		p
	n (30)	%	n (30)	%	
Gender					
Male	7	23,3	7	23,3	1,00
Female	23	76,7	23	76,7	
Age (years old), Mean ± SD	40,23 ± 9,98		39,6 ± 9,76		0,805
21 – 30	5	16,7	7	23,3	
31 – 40	11	36,7	9	30	
41 – 50	8	26,7	8	26,7	
51 – 60	6	20	6	20	
TRAb (pg/mL) Mean ± SD	288,25±267,6	-	144,21±21,13	-	0,000

Prevalence of FOXP3 polymorphisms in GD patients

Aim of this study is to prove the existence of FOXP3 gene promoter polymorphism in patients with Graves' disease. The sequence examination on the FOXP3 gene produces 5 SNP found on the FOXP3 gene promoter. In 30 subjects with GD, all subjects had mutations (100%) with each having 1,2 or 3 mutations with different SNPs while 26 control subjects had mutations (86.7%) with different SNPs.

Polymorphism of SNP -924A/G and -1383C/T in GD patients

Table 2 shows that there is no polymorphism in SNP rs2232364 which is indicated by the finding of CC allele (Wild type) in both patient and control group. Polymorphism SNP 924A/G was higher in Graves' patients with a percentage of 61.1% versus 38.9% in the control group, with heterozygous mutations (AG) being the most common. From the results of the SNP -924A/G statistical test, there was no difference between the Graves patient group and the control ($p > 0.05$).

Table 2. Polymophisms of SNP 924A/G and 1383C/T in GD patients and control

SNP	Allele	Allele Frequency				Total		p
		Graves' patients		Control				
		f	%	f	%	f	%	
rs2232365	AA	11	47,8	12	52,2	23	100	
(924 A/G)	AG	11	61,1	7	38,9	18	100	0,678
	GG	8	50	8	50	16	100	
rs2232364	CC	30	52,6	27	47,4	57	100	Cannot be
(1383 C/T)	CT	0	0	0	0	0	0	analyzed
	TT	0	0	0	0	0	0	

Deletion in FOXP3 Gene Promotor Sequence

On this study, we are also found that there is deletion found of new SNPs which not yet registered in GeneBank NCBI located on base 49,259,740 marked with red box. This deletion was found in only 3 samples of Graves' patient group, and was not found in the control group. In this SNP, a basic change occurs which is supposed to be a T allele, being lost on samples C15, C16 and C17 as shown in figure 1. These samples were female, in which two of the three samples had higher TRAb levels than mean of TRAb in GD patients with TRAb level of 964,14 ng/ml and 816,26 ng/ml compared to 288,25 ± 267 ng/ml.

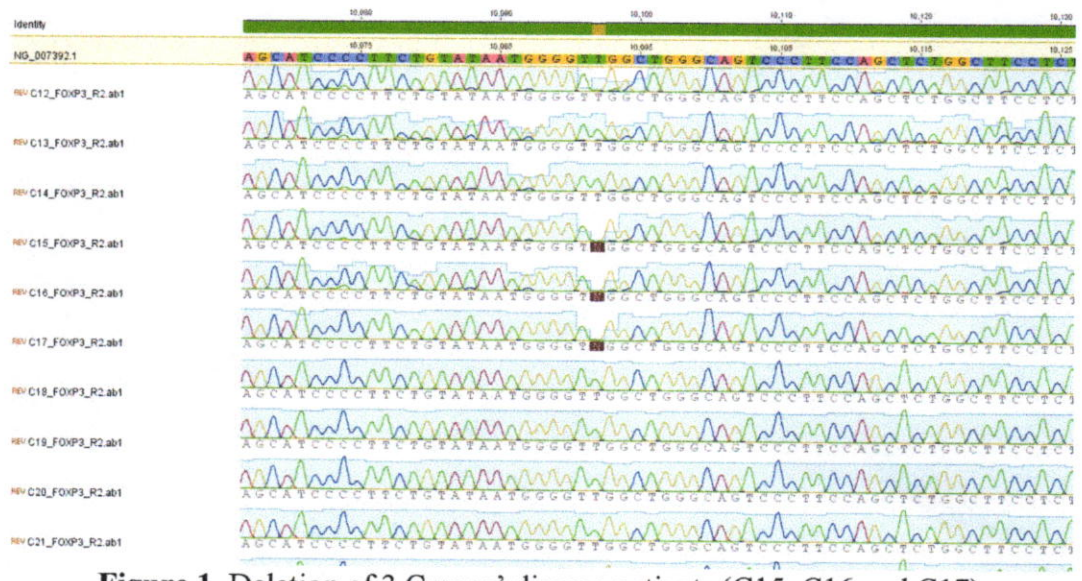

Figure 1. Deletion of 3 Graves' disease patients (C15, C16 and C17)

Polymorphism of SNP - 2383C/T, - 3279C/A and - 3499A/G in GD patients

From this study, it is seen that these three SNPs had polymorphisms characterized by the discovery of homozygous and heterozygous polymorphism/mutants. Table 3 shows that in Graves' patients with SNP polymorphism 3499 A/G has the highest percentage in heterozygous mutant polymorphism (AC). There was no statistically significant difference between the three groups ($p > 0.05$). Polymorphism SNP 3279 C/A heterozygote mutant (AC) has the highest percentage. Statistically, there was no significant difference ($p > 0,05$). Furthermore, in SNP rs3761549 the percentage of heterozygous mutants (CT) is higher than other polymorphism motifs. From the results of statistical tests conducted, there is no significant difference ($p > 0.05$).

Table 3. Polymophisms of SNP - 2383C/T, - 3279C/A and - 3499A/G in GD patients and control.

SNP	Allele	Allele Frequency				Total		p
		Graves' patients		Control				
		f	%	f	%	f	%	
rs3761547	**AA***	22	51,2	21	48,8	43	100	
(3499 A/G)	AG	6	66,7	3	33,3	9	100	0,586
	GG	2	40	3	60	5	100	
rs3761548	**AA***	4	40	6	60	10	100	
(3279 C/A)	AC	8	72,7	3	27,3	11	100	0,283
	CC	18	50	18	50	36	100	
rs3761549	**CC***	22	52,4	23	47,6	45	100	
(2383 C/T)	CT	6	60	4	40	10	100	0,764
	TT	2	40	3	60	5	100	

* = wild type

Association between FOXP3 polymorphisms and GD

The result of complete analysis of polymorphism of SNP FOXP3 gene promoter with incidence of Graves' disease can be seen in table 4 Table 4 shows that based on PCR direct-sequencing-method result in Graves' patients found SNP 924 A/G polymorphism more common in the Graves' than controls, with a percentage of 54.3% versus 45.7%, but statistical tests showed no difference between both groups with $p > 0.05$. The polymorphism of SNP 3499 A/G, SNP 3279 C/A and SNP 2383 C/T were found to be similar between GD group and controls with 50% in percentage, no significant differences were found between the two groups. Based on Table 5.6, it was seen in the Graves group of patients with SNP 924 A/G polymorphism and 3279 C/A higher than those without polymorphism and no SNP polymorphism 1383 C/T found on this study.

Table 4. Polymorphisms of FOXP3 Gene Promoter in GD Patients

SNP	Graves' disease				Total		p
Polymorphisms	Yes		No				
rs2232365	f	%	f	%	f	%	
Presence	19	54,3	16	45,7	35	100	0,6
Absence	11	44	14	56	25	100	
rs2232364							
Presence	0	0	0	0	0	0	-
Absence	30	50	30	50	60	100	
rs3761547							
Presence	8	50	8	50	16	100	1,00
Absence	22	50	22	50	44	100	
rs3761548							
Presence	26	50	26	50	52	100	1,00
Absence	4	50	4	50	8	100	
rs3761549							
Presence	8	50	8	50	16	100	1,00
Absence	22	50	22	50	44	100	

DISCUSSION

Characteristics of study subjects in the form of sex and age did not differ significantly between the GD and control group. In this study, the age range between 31-40 years and female gender was the age and sex group which mostly found in GD patients. This is consistent with the American Thyroid Association's data which explains that although Graves' disease can occur in all age groups, it is more common in women than in men with a 7-8: 1 ratio. Like most other autoimmune diseases, female sex is the most frequent group of autoimmune diseases. It is suspected that the role of estrogen in stimulating antibody production and autoantibodies through B cells. Estrogen also increases the levels of IL-4, IL-10 and TGF-β as well as expression of CD80 and FOXP3, which further increases CTLA-4 activity and T-reg cell populations [8].

The FOXP3 gene is located on chromosome Xp11.23 with a length of 1,146 bp, containing 11 exons coding 431 amino acids. The FOXP3 gene is the main gene that responsible for development of regulatory T cells, which comprise the promoter region, first intron (consisting of CNS1 and CNS2), and intron after the first exon coding (CNS3). The FOXP3 promoter region is the area most responsible for T-regulator cell differentiation. Mutations in the FOXP3 gene promoter region may lead to the dysfunction and formation of auto aggressive lymphocyte clones that contribute to the occurrence of autoimmunity. In the FOXP3 gene promoter region there are 5 SNPs that may affect the expression of the FOXP3 gene, ie - 924A / G (rs2232365), - 1383C / T (rs2232364), - 3499A / G (rs3761547), - 3279C / A (rs3761548) and - 2383C / T (rs3761549). Of all the FOXP3 mutations that trigger autoimmunity, manifestations of the thyroid gland are the most frequently affected [9,10].

In this study, it was proved that 4 of 5 SNP polymorphisms of FOXP3 gene promoter, ie - 924A / G (rs2232365), - 3499A / G (rs3761547), - 3279C / A (rs3761548) and - 2383C / T (rs3761549) found in GD. The polymorphism of SNP rs3761548 and rs2232365 were the most common SNPs in this study in which of 26 subjects experienced polymorphism SNP rs3761548 with the finding of heterozygous mutants (AC) of 72.7% and homozygotes (CC) of 50% SNP rs3761548. While SNP rs2232365 of 19 subjects found heterozygous mutant polymorphism (AG) of 61.1% and homozygous mutants by 50%. From the statistical test results, there was no significant difference between the two SNPs with the control group with $p > 0.05$. Polymorphism SNP rs3761547 and rs3761549 were found to be 26.7% of the 30 subjects of Graves' patients. In this study, no SNP rs2232364 polymorphism was found in both Graves and control groups. In this study, we also found 1 new SNP that experienced deletion in 3 samples of Graves' patients. The deletions in the 49,259,740-base order found are not listed on the Gene Bank NCBI. The three samples were female sex, of which two of the three samples had higher TRAb levels than the mean Graves' average of 890.20 ng/ml.

Owen *et al* study in 2006, conducted 633 Graves' patients in the United Kingdom showed no finding of polymorphism of SNP rs2232365 and rs3761549 gene promoters, but there is a polymorphism in the CATTC minor haplotype suspected of contributing to Graves' disease. Inoue *et al* in 2010 research conducted in Japan showed similar results with this study, where SNP polymorphism rs3761548 was also found in Graves patients with the most commonly found CC genotype of 71%, followed by an AC genotype of 17.7% (Inoue et al., 2010). In addition to SNP rs3761548, Inoue et al, also examined two other SNPs namely SNP rs3761547 and rs3761549. However, unlike this study, SNP polymorphism rs3761547 was found as much as 43.1% and SNP rs3761549 as much as 37.9% greater than polymorphism found in this study that is 26.7%. Yu *et al* in 2017 who conducted a study of 534 Graves' populations in China showed the acquisition of SNP polymorphism rs3761548 and rs3761549 with percentages respectively of 93% and 43%, greater than this study of 86.7% and 26.7% [11,12,13].

In this study, no difference was found between polymorphism of SNP FOXP3 gene promoter in patients with Graves' disease with control with p> 0.05 on each SNP. This is consistent with the study of Owen *et al* in 2008 in the United Kingdom population showing that there is no difference in the relationship between FOXP3 gene polymorphisms and healthy controls, while different results are shown by the study of Zheng *et al.*, 2015 which examines Graves' populations in Han Chinese populations which shows there is a difference between SNP polymorphism rs3761548 and healthy control [14,15].

Many factors affect the incidence of Graves' disease, both other genetic and environmental factors. The study of Eliana et al., 2017 shows the presence of CTLA4 gene polymorphs of 49 codons 17 exon 1 in Graves patients between relapse and non-relapse patints. Cytotoxic T-lymphocyte-associated Protein-4 (CTLA4) is one of the markers on the surface of T-regulator cells that also play a role in immune homeostasis along with the FOXP3 gene present in intracellular T-regulators. Recent research also shows that environmental factors can also interact with certain genes that lead to susceptibility to Graves' disease through epigenetic modulation, such as the occurrence of histone modification. Research of Yan et al., 2015 found that histone H4 acetylation levels decreased in Graves patients, while Histone deacetylase 1 (HDAC1) and Histone deacetylase 2 (HDAC2) levels were very high which showed that histone modification or modification occurred in Graves patients. However, the study of the role of histone modification in Graves 'own disease is still largely unrelated to the histone-modifying gene of Sirtuin1 (SIRT1) suspected of having an effect on histone modification in Graves' patients. From some research that has been done on FOXP3 polymorphism, SNP rs2232365 also found in some other autoimmune diseases such as Chron disease, Addison's disease and psoriasis. While SNP rs3761548 is also found in allergic rhinitis, atopy, psoriasis and systemic lupus erythematosus. The discovery of this FOXP3 gene polymorphism in a number of other autoimmune diseases suggests that the FOXP3 gene plays a role in maintaining immune system balance and controlling T cell activity and effector T cell function [16,17,18].

CONCLUSION

In conclusion, we prove that there are polymorphisms of FOXP3 gene promoter SNP - 924A/G, -2383C/T, - 3279C/A and - 3499A/G and no polymorphism SNP - 1383C/T found on this study. Furthermore, subjects carrying SNP -924A/G and -3279C/A FOXP3 polymorphisms are more susceptible to GD which mostly found on GD patients.

Declaration of conflicting interests

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