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Association of functional polymorphisms related to the transcriptional level of *FOXP3* with prognosis of autoimmune thyroid diseases

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Summary

The severity of Hashimoto's disease (HD) and intractability (or inducibility to remission) of Graves' disease (GD) varies among patients. Forkhead box P3 (FoxP3) is a crucial regulatory factor for the development and function of regulatory T (T_{reg}) cells, and deficiency of the FoxP3 gene (*FOXP3*) suppresses the regulatory function of T_{reg} cells. To clarify the association of the functional polymorphisms of the *FOXP3* with the prognosis of GD and HD, we genotyped −3499A/G, −3279C/A and −2383C/T polymorphisms in *FOXP3* gene obtained from 38 patients with severe HD, 40 patients with mild HD, 65 patients with intractable GD, in whom remission was difficult to induce, 44 patients with GD in remission and 71 healthy volunteers. The −3279CA genotype was more frequent in patients with GD in remission than in patients with intractable GD, and the −3279AA genotype, which correlates to defective transcription of *FOXP3*, was absent in patients with GD in remission. The −2383CC genotype was more frequent in patients with severe HD than in those with mild HD. In conclusion, the −3279A/C polymorphism is related to the development and intractability of GD and the −2383CC genotype to the severity of HD.

Keywords: disease intractability, disease severity, FoxP3, regulatory T cell, single nucleotide polymorphism

Introduction

Autoimmune thyroid diseases (AITDs), such as Hashimoto's disease (HD) and Graves' disease (GD), are archetypes for organ-specific autoimmune diseases [1,2]. The severity of HD and intractability (that is, inducibility to remission) of GD vary among patients. Some patients develop hypothyroidism in early life, whereas others maintain a euthyroid state into old age [3,4]. Some patients with GD achieve remission through medical treatment, while others do not. However, the severity of HD and intractability of GD are difficult to predict at the first diagnosis. We have reported previously that hypothyroidism caused by severe destruction of thyroid tissue is more likely to develop in HD patients with a genetically higher production of interferon (IFN)-γ [5] or a genetically lower production of interleukin (IL)-4 [6].

Regulatory T (T_{reg}) cells are known to play an important role in immune regulation. In a mouse model of GD, T_{reg} cells appear to be crucial in the pathogenesis of GD [7]. Moreover, we have reported previously an apoptosis-induced decrease in the proportion of intrathyroidal T_{reg} cells in patients with AITD [8]. Therefore, we consider that T_{reg} may be involved in preventing the development of AITDs.

Forkhead box P3 (FoxP3) is a major regulatory factor for the development and functioning of T_{reg} cells [9]. FoxP3 controls T_{reg} function through co-operation with nuclear factor of activated T cells (NFAT) [10], and deficiency of the FoxP3 gene (*FOXP3*) impairs the suppressor function of T_{reg} cells [11]. Some microsatellites in *FOXP3* were associated with susceptibility to AITD in Caucasians, but these associations were not observed in the Japanese population [12]. There are five single nucleotide polymorphisms (SNP) in the promoter region of *FOXP3*. These are −924A/G (rs2232365), −1383C/T (rs2232364), −2383C/T (rs3761549), −3279C/A (rs3761548) and −3499A/G (rs3761547) polymorphisms [13], and they may affect the expression of *FOXP3*. In the present study, we focused upon −2383C/T, −3279C/A and −3499A/G polymorphisms, as these are common in the Japanese population according to the National Center for Biotechnology Information (NCBI) SNP (http://www.ncbi.nlm.nih.gov/snp/) and Japanese (J)-SNP databases (http://snp.ims.u-tokyo.ac.jp/). The AA genotype of the −3279C/A polymorphism causes the loss of binding with some

Table 1. Clinical characteristics of groups of patients with autoimmune thyroid disease at the time of sampling and the age of onset.

		Graves' disease		Hashimoto's disease	
		Presence of the past clinical history of thyrotoxicosis with elevated TRAb		Presence of the diffuse goitre and the positive TgAb and/or McAb	
	Controls	Intractable	In remission	Severe	Mild
Female/male (*n*)	71 (61/10)	65 (57/8)	44 (40/4)	38 (33/5)	40 (34/6)
Age of onset (years)	44·1 ± 12·2†	35·2 ± 14·7	29·0 ± 12·7	37·3 ± 11·1	58·7 ± 8·9†
(range)	(21–67)†	(10–49)	(15–66)	(10–49)	(50–79)†
FreeT4 (ng/dl)	n.d.	1·38 ± 0·68	1·35 ± 0·63	1·42 ± 0·24	1·43 ± 0·61
FreeT3 (pg/ml)	n.d.	4·12 ± 3·42	3·20 ± 1·62	2·82 ± 0·38	3·28 ± 1·42
TSH (μU/ml)	n.d.	2·59 ± 2·63	2·43 ± 3·47	1·84 ± 2·04	1·99 ± 1·43
TRAb (IU/l)	< 1·0	7·3 ± 12·5	< 1·0	< 1·0	< 1·0
(range)		(1·1–71·0)			
TgAb ($2^n \times 100$)	Negative	2·9 ± 0·6	1·7 ± 1·5	7·7 ± 3·1*	1·7 ± 2·7
McAb ($2^n \times 100$)	Negative	4·6 ± 2·6	5·3 ± 2·2	6·1 ± 2·7*	2·4 ± 0·8
Treatment	None	Methimazole or PTU	None	L-thyroxine	None

*$P < 0{\cdot}01$ *versus* mild Hashimoto's disease. †Age at the time of sampling. Data are expressed as mean ± standard deviation. n.d.: not determined; T4: thyroxine; T3: triiodothyronine; TSH: thyrotropin; PTU: propylthiouracil; TRAb: anti-thyrotropin receptor antibody; McAb: anti-thyroid microsomal antibody; TgAb: anti-thyroglobulin antibody.

transcription factors, such as E47 and C-Myb, leading to defective transcription of *FOXP3* [14]. Moreover, the A allele of this polymorphism is associated with a dramatic reduction in luciferase activity compared with the C allele [14]. Conversely, there is no report on the functional effects of −2383C/T and −3499A/G polymorphisms on gene expression.

In this study, we genotyped these three polymorphisms in Japanese patients to clarify their effects on the development and prognosis of AITDs.

Materials and methods

Subjects

Genomic DNA was obtained from 38 patients with HD who developed moderate to severe hypothyroidism before 50 years of age and were treated daily with at least 1·5 μg thyroxine per kg body weight (severe HD), and from 40 untreated, euthyroid patients with HD who were more than 50 years of age (mild HD). All patients with HD were positive for anti-thyroid microsomal antibody (McAb) or anti-thyroglobulin antibody (TgAb) and all patients with mild HD had palpable diffuse goitre. Also examined were 65 euthyroid patients with GD who had been treated with methimazole or propylthiouracil for at least 5 years and were still positive for TRAb (intractable GD), 44 GD patients in remission who had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication (GD in remission) and 71 healthy volunteers (control subjects), who were euthyroid and were negative for thyroid autoantibodies, were also examined. All patients and control subjects were Japanese and unrelated. All patients were followed-up closely for more than 5 years as out-patients at our thyroid clinic. Patients with AITD did not suffer from other autoimmune diseases. The clinical characteristics of groups of patients at the time of sampling and the ages of onset were shown in Table 1. The titres of TgAb were significantly higher in patients with severe HD than in those with mild HD, as our previous report [15].

Genomic DNA was isolated from ethylenediamine tetraacetic acid (EDTA)-treated peripheral blood mononuclear cells with a commercially available kit (DrGenTLE™; Takara Bio Inc., Shiga, Japan). Written informed consent was obtained from all patients and controls, and the study protocol was approved by the Ethics Committee of Osaka University.

Genotyping of −2383C/T polymorphism

The target sequence of *FOXP3* gene was amplified using polymerase chain reaction (PCR). The forward primer was 5′-GCCTGGCACTCTCAGAGCTT-3′, the reverse primer was 5′-GTCTGTGGAGGCTCCGAACA-3′. The protocol for the PCR was as follows: 95°C for 3 min and 30 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min and a single final extension at 72°C for 10 min. The PCR product [942 base pairs (bp)] was digested by addition of *Bsr*I and incubation at 65°C for 6 h.

Genotyping of −3279C/A polymorphism

The target sequence of the *FOXP3* gene was amplified using PCR. The forward primer was 5′-CCTCTCCGTGCTCAGT GTAG-3′, the reverse primer was 5′-CTCACCTAGCCCAGC TCTTG-3′. The protocol for the PCR was as follows: 94°C for 5 min and 30 cycles of denaturing at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s and a single

final extension at 72°C for 7 min. The PCR product (300 bp) was digested by addition of *Pst*I and incubation at 37°C for 6 h.

Genotyping of −3499A/G polymorphism

The target sequence of the *FOXP3* gene was amplified using PCR. The forward primer was 5′-CTCTGGCTCTCCATG CATGT-3′, the reverse primer was 5′-TGCAGGGCTTCAA GTTGACAG-3′. The protocol for the PCR was as follows: 94°C for 5 min and 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s and a single final extension at 72°C for 7 min. The PCR product (158 bp) was digested by addition of *Pvu*II and incubation at 37°C for 6 h.

Thyroid function and autoantibodies

The serum concentration of free T4 (FT4) was measured with a commercial radioimmunoassay kit (Eiken Chemical Co. Ltd, Tokyo, Japan). The normal range of serum FT4 is 1·0–1·6 ng/dl (12·9–20·6 pmol/l). The serum concentration of free T3 (FT3) was measured with a radioimmunoassay kit (Japan Kodak diagnostic Co. Ltd, Tokyo, Japan). The normal range of serum FT3 is 2·4–4·6 pg/ml (3·8–7·2 pmol/l). The serum TSH concentration was also measured with a radioimmunoassay kit (Daiichi Radioisotope Laboratories Ltd, Tokyo, Japan). The normal range of serum TSH is 0·6–5·4 μU/ml. TgAb and McAb were measured with a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titre of > 1:100 was considered positive. Serum level of TRAb was determined with an enzyme-linked immunosorbent assay (ELISA) (third-generation) (Cosmic Co., Tokyo, Japan). The normal value of TRAb was less than 1·0 IU/l.

Statistical analysis

We used χ^2 tests and Fisher's exact test to evaluate the significance of differences in frequencies of genotype and allele among the subject groups. Student's *t*-test was used to analyse difference in goitre size. A Mann–Whitney *U*-test was used to analyse differences in serum titres of McAb, TgAb and TRAb. Data were analysed with JMP8 software (SAS Institute, Inc., Tokyo, Japan). Probability values of less than 0·05 were considered significant.

Results

−2383C/T polymorphism

The frequency of this polymorphism did not differ between normal subjects and each group of patients with HD and GD (Table 2).

The CC genotype was significantly frequent in the patients with severe HD than in those with mild HD [$P = 0{\cdot}0220$, odds ratio (OR) = 3·01] (Table 2). However, we found no difference in genotypes and allele frequencies of this polymorphism between in the patients with intractable GD and those with GD in remission (Table 2).

−3279C/A polymorphism

Genotypic distribution was significantly different between intractable GD and GD in remission groups ($P = 0{\cdot}0016$) and the AA genotype was found in 11·3% of patients with intractable GD, whereas the proportion of AA genotype was 0% in patients with GD in remission. Moreover, the frequency of CA genotype was significantly higher in patients with GD in remission than in those with intractable GD ($P = 0{\cdot}0048$, OR = 3·71) (Table 2).

Conversely, we found no significant difference in the genotype and allele frequencies between two HD groups (Table 2).

−3499A/G polymorphism

We found no difference in genotype and allele frequencies of −3499 A/G polymorphism among any groups (Table 2).

Association between each polymorphism and levels of autoantibody

We found no association each of TRAb levels, McAb titres and TgAb titres with examined polymorphisms (data not shown).

Discussion

Because T_{reg} cells play a role in suppressing the development of autoimmune diseases by inhibiting autoreactive T cells [16] and *FOXP3* is a master gene of T_{reg} cells, we expected that *FOXP3* genotypes related to a lower expression of FoxP3 would be frequent in AITD patients.

In the case of the *FOXP3* −3279C/A polymorphism, the A allele correlates with a reduction in FoxP3 expression [14]. Interestingly, we determined that 11·3% of patients with intractable GD had the −3279AA genotype, which shows the lowest production of FoxP3 among the three genotypes of this polymorphism [14], whereas this genotype was absent in patients with GD in remission (Table 2). Therefore, GD patients with the −3279AA genotype may have T_{reg} cells with weak suppressor activity, and as FoxP3 controls T_{reg} function [10] it may be difficult for such patients to achieve remission. These results are consistent with our previous report, indicating a decrease in the proportion of intrathyroidal T_{reg} cells in patients with intractable GD [8]. Therefore, the −3279AA genotype may contribute to the severity of the immune response. Surprisingly, in addition, the −3279CA genotype (heterozygote) was more frequent in patients with GD in remission than in patients with intractable GD ($P = 0{\cdot}0048$,

Table 2. Genotype and allele frequencies of the forkhead box P3 polymorphisms in patients with Graves' disease, Hashimoto's disease and in control subjects.

			Graves' disease			Hashimoto's disease		
		Control	Intractable	In remission		Severe	Mild	
−2383C/T	CC	38 (61·2%)	36 (62·1%)	20 (69·0%)		23 (67·6%)	16 (41·0%)	
	CT	19 (30·7%)	16 (27·6%)	8 (27·6%)	n.s.†	9 (26·5%)	20 (51·3%)	n.s.‡
	TT	5 (8·1%)	6 (10·3%)	1 (3·4%)		2 (5·9%)	3 (7·7%)	
	CC	38 (61·2%)	36 (62·1%)	20 (69·0%)	n.s.†	23 (67·6%)	16 (41·0%)	$P = 0{\cdot}0220$‡
	CT + TT	24 (38·8%)	22 (37·9%)	9 (31·0%)		11 (32·4%)	23 (59·0%)	OR = 3·01 (1·15–7·86)
	C allele	95 (76·6%)	88 (75·9%)	48 (82·8%)	n.s.†	55 (79·7%)	52 (66·7%)	n.s.‡
	T allele	29 (23·4%)	28 (24·1%)	10 (17·2%)		13 (20·3%)	26 (33·3%)	
−3279C/A	CC	58 (81·7%)	44 (71·0%)	20 (55·6%)		31 (81·5%)	26 (76·5%)	
	CA	8 (11·3%)	11 (17·7%)	16 (44·4%)	$P = 0{\cdot}0016$†	5 (13·2%)	7 (20·6%)	n.s.‡
	AA	5 (7·0%)	7 (11·3%)	0 (0%)		2 (5·3%)	1 (2·9%)	
	AA	5 (7·0%)	7 (11·3%)	0 (0%)	$P = 0{\cdot}0449$†§	2 (5·3%)	1 (2·9%)	n.s.‡§
	CA + CC	66 (93·0%)	55 (88·7%)	36 (100%)		36 (94·7%)	33 (97·1%)	
	CA	8 (11·3%)	11 (17·7%)	16 (44·4%)	$P = 0{\cdot}0048$†	5 (13·2%)	7 (20·6%)	n.s.‡
	AA + CC	63 (88·7%)	51 (82·3%)	20 (55·6%)	$OR = 3{\cdot}71$ (1·47–9·36)	33 (86·8%)	27 (79·4%)	
	A allele	18 (12·7%)	25 (19·7%)	16 (22·2%)	n.s.†	9 (12·5%)	9 (14·5%)	n.s.‡
	C allele	124 (87·3%)	99 (80·3%)	56 (77·8%)		63 (87·5%)	53 (85·5%)	
−3499A/G	AA	46 (65·7%)	37 (56·9%)	32 (72·7%)		20 (62·5%)	19 (47·5%)	
	AG	20 (28·6%)	22 (33·9%)	11 (25·0%)	n.s.†	11 (34·4%)	18 (45·0%)	n.s.‡
	GG	4 (5·7%)	6 (9·2%)	1 (2·3%)		1 (3·1%)	3 (7·5%)	
	AA	46 (65·7%)	37 (56·9%)	32 (72·7%)	n.s.†	20 (62·5%)	19 (47·5%)	n.s.‡
	AG + GG	24 (34·3%)	28 (43·1%)	12 (27·3%)		12 (37·5%)	21 (52·5%)	
	A allele	112 (80·0%)	96 (73·8%)	75 (85·2%)	n.s.†	51 (79·7%)	56 (70·0%)	n.s.‡
	G allele	28 (20·0%)	34 (26·2%)	13 (14·8%)		13 (20·3%)	24 (30·0%)	

Analysed by χ^2 tests or †intractable Graves' disease *versus* Graves' disease in remission. ‡Severe Hashimoto's disease *versus* mild Hashimoto's disease. §Fisher's exact test. n.s.: not significant; OR: odds ratio (95% confidence interval).

OR = 3·71) (Table 2). Although the functional significance of the heterozygous −3279AC genotype of *FOXP3* is still unclear, this heterozygosity may itself have unknown but considerable effects on GD pathogenesis. This possibility is supported by the previous finding that the *FOXP3* −3279CA genotype was also more frequent in patients with allergic rhinitis [17] which, like GD, is caused by antibodies.

For the −2383C/T polymorphism, the distribution of alleles and genotypes did not differ between normal subjects and GD patients in this study. These distributions also did not differ between normal subjects and GD patients in a UK population [18], suggesting that this polymorphism may have no effect on the pathogenesis of GD. However, in our study, the −2383CC genotype was significantly more frequent in patients with severe HD than in those with mild HD ($P = 0{\cdot}0220$, OR = 3·01) (Table 2). Because the functional differences of each allele in this polymorphism are still unclear, we used p-Match software [19] to predict the binding sites, and attempted to estimate the effect of this polymorphism. The result of this search indicated that the sequence of the −2383T allele may be essential for the binding of a transcriptional factor Yin-Yang-1 (YY1) with *FOXP3*. The consensus YY-1 motif is CATNTWN ('N' representing any nucleotide and 'W' representing A or T); the −2383T allele creates the DNA sequence CAGCTAG and the −2383C allele creates CAGCCAG. As the number of mismatched bases compared with the YY-1 motif is lower for the T allele of this polymorphism than for the C allele, YY-1 may bind this sequence more strongly in the −2383T allele than in the C allele. In addition, YY-1 also binds to the promoter region and enhances promoter activity in *IL4* [20]. Therefore, we assumed that individuals with the CC genotype of the −2383C/T polymorphism might exhibit a lower expression of FoxP3, resulting in weak suppressor function of T_{reg} cells. This decrease in regulatory function may increase the activity of autoreactive T cells and may cause severe autoimmune destruction of thyroid tissue in HD patients with the CC genotype. Although some interesting significances were found in this study, it may be necessary to examine a larger number of samples to perform a more detailed analysis, such as haplotype analysis, using these polymorphisms. This limitation of sample numbers was due to very strict criteria for selection of samples to exclude borderline cases and enhance the study reliability.

In conclusion, functional polymorphisms of *FOXP3* are associated with the prognosis of AITDs. The −3279A/C poly-

morphism is related to the development and intractability of GD, and the CC genotype of the –2383C/T polymorphism is related to the severity of HD.

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Disclosure

The authors have no financial conflicts of interest.

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