Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2007.03575.x

The +869T/C polymorphism in the transforming growth factor-β1 gene is associated with the severity and intractability of autoimmune thyroid disease

H. Yamada,* M. Watanabe,* T. Nanba,* T. Akamizu† and Y. Iwatani*

**Department of Biomedical Informatics, Division of Health Sciences, Osaka University Graduate School of Medicine, Osaka, and †Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan*

Accepted for publication 14 November 2007
Correspondence: Y. Iwatani, Department of Biomedical Informatics, Division of Health Sciences, Osaka University Graduate School of Medicine, Yamadaoka 1-7, Suita, Osaka 565-0871, Japan.
E-mail: iwatani@sahs.med.osaka-u.ac.jp

Summary

The severity of Hashimoto's disease (HD) and the intractability of Graves' disease (GD) vary among patients. To clarify whether the +869T/C polymorphism in the transforming growth factor-β1 (TGF-β1) gene, which is associated with TGF-β1 expression, is involved in the intractability of GD and severity of HD, we genotyped the TGF-β1 +869T/C polymorphism by polymerase chain reaction–restriction fragment length polymorphism method in genomic DNA samples from 33 patients with HD who developed hypothyroidism before they were 50 years old (severe HD) and 30 untreated, euthyroid patients with HD who were older than 50 years (mild HD). We also examined 48 euthyroid patients with GD who had been under treatment and were still positive for anti-thyrotropin receptor antibodies (intractable GD), 20 euthyroid patients with GD in remission and 45 healthy controls. The frequency of the T allele and the TT genotype were higher in patients with severe HD than in those with in mild HD. In contrast, the frequency of the CC genotype was higher in patients with intractable GD than in patients with GD in remission. In conclusion, the +869T/C polymorphism in the TGF-β1 gene is associated with the severity and intractability of autoimmune thyroid disease.

Keywords: autoimmune thyroid disease, cytotoxic T cell, disease severity, single nucleotide polymorphism, TGF-β1

Introduction

Autoimmune thyroid diseases (AITD), such as Graves' disease (GD) and Hashimoto's disease (HD), are archetypes for organ-specific autoimmune diseases [1,2]. The intractability of GD and the severity of HD vary among patients. Some patients with GD achieve remission through medical treatment, but others do not. Most patients with HD maintain a lifetime euthyroid state without any medical treatment, whereas others become hypothyroid. Although the immunological differences that underlie the differences in intractability or severity remain unclear, we have reported previously that $CD8^+$ $CD25^+$-activated cytotoxic T lymphocytes are involved in the severity of HD [3] and that the frequency of the T allele of the +874A/T polymorphism in the interferon (IFN)-γ gene, which is associated with high IFN-γ production, is higher in patients with severe HD than in those with mild HD [4]. It was suggested that increased IFN-γ, a major cytokine produced by T helper type 1 (Th1) cells [5], activates cytotoxic T lymphocytes and enhances cell-mediated cytotoxicity in severe HD [3,4].

Transforming growth factor-β1 (TGF-β1) is important to induce and maintain tolerance against several self-antigens [6]. The human TGF-β1 gene, which is located on chromosome 19q13, contains seven exons that give rise to a precursor protein of 390 amino acids [7]. TGF-β1 inhibits the proliferation of T and B cells and the generation of T cell cytotoxicity [8,9]. Furthermore, TGF-β1 also inhibits cytokine production and decreases the expression of human leucocyte class II antigens (HLA) induced by IFN-γ [10].

There is a polymorphism in the TGF-β1 gene, +869T/C in the region encoding the signal peptide, that results in a Leu to Pro substitution at amino acid position 10. The C allele (Pro) of this polymorphism shows a 2·8-fold higher secretion of TGF-β1 than the T allele (Leu) in HeLa cells [11], and the C allele is also associated with high serum concentrations of TGF-β1 [12]. This difference in serum concentration is more apparent in the CC homozygote than in the CT and TT homozygotes. Although weak associations were reported between the TGF-β1 +869T/C polymorphism and susceptibilities to rheumatoid arthritis [13] and systemic sclerosis [14], no association was observed for AITD [15].

Furthermore, the serum concentration of TGF-β1 is not associated with susceptibility to GD [16].

Because TGF-β1 modulates the immune system systematically, and its effect is not specific to the thyroid, this polymorphism may be associated with the intractability of GD and the severity of HD rather than the susceptibility to these diseases. Therefore, in the present study we investigated the association between this polymorphism and the intractability and severity of AITD.

Subjects and methods

Subjects

We screened for the +869T/C polymorphism in 48 euthyroid patients (five men and 43 women; 50·2 ± 16·1 years old) with GD who had been treated with 5–15 mg/day of methimazole for at least 5 years and were still positive for anti-thyrotropin receptor antibody (TRAb) (intractable GD) and in 20 patients (one man and 19 women; 48·9 ± 13·0 years old) with GD in remission who had maintained a euthyroid state and were negative for TRAb for more than 5 years without medication (GD in remission). We also examined 33 patients (four men and 29 women; 52·1 ± 9·9 years old) with HD who developed moderate to severe hypothyroidism before the age of 50 years and were treated daily with at least 1·5 μg thyroxine per kg body weight (severe HD) and 30 untreated, euthyroid patients (one man and 29 women; 56·2 ± 6·6 years old) with HD who were older than 50 years (mild HD). All patients with HD were positive for anti-thyroid microsomal antibody (McAb). Because the prevalence of hypothyroidism caused by autoimmune thyroid destruction increases with age [17], we classified HD patients who developed hypothyroidism before the age of 50 years, as severe HD patients and euthyroid HD patients who had not developed hypothyroidism by 50 years of age as mild HD patients. Patients with transient hypothyroidism were excluded. All patients did not have any other autoimmune diseases and autoantibodies. Forty-five healthy volunteers (five men and 40 women; 47·6 ± 12·2 years old) who were euthyroid and negative for thyroid autoantibodies were included as control subjects. All patients and control subjects were Japanese and unrelated. Written informed consent was obtained from all patients and control subjects, and the study protocol was approved by the Ethics Committee of Osaka University (Osaka, Japan).

Genotyping of the +869T/C polymorphism in the TGF-β1 gene

Genomic DNA was isolated from 1 ml of ethylenediamine tetraacetic acid (EDTA)-treated peripheral blood with a commercially available kit (Dr GenTLE™, Takara Bio Inc., Shiga, Japan). Genotyping was performed with the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. Briefly, isolated genomic DNA was amplified with primers 5′-AGGTT ATTTCCGTGGGATAC-3′ (forward) and 5′-ATAGTCTT GCAGGTGGATAG-3′ (reverse). Amplification conditions consisted of one cycle of 94°C for 4 min followed by 35 cycles of 95°C for 30, 50°C for 30 and 72°C for 1 min. The PCR products [300 base pairs (bp)] were then digested with restriction enzyme MspA1 I (New England Biolabs, Beverly, MA, USA) for 4 h at 37°C. The T allele was recognized by two fragments of 244 and 64 bp, while the C allele showed three bands of 232, 66 and 12 bp. DNA fragment length polymorphisms were visualized by UV transillumination after electrophoretic separation on an 8% polyacryamide gel. Genotyping of this polymorphism was confirmed by the direct sequencing method and PCRs were repeated to confirm initial results in all samples.

Assay of thyroid function and autoantibody levels

The serum concentration of free T4 (FT4) was measured with a 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. Anti-thyrotropin receptor antibodies (TgAb) and McAb were measured with a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titre of >1 : 100 was considered positive. Serum TRAb was measured with a radioreceptor assay (Cosmic Co., Tokyo, Japan); results are expressed as percentage inhibition of binding of labelled TSH. The normal value is less than 10%.

Statistical analysis

All patients and control subjects were categorized into three groups on the basis of the +869T/C genotype: TT, CT or CC. We used the χ^2 test to evaluate the significance of differences between genotype frequencies in the two GD groups, and between those in the two HD groups. The number of T and C alleles was calculated in each patient group and in the control group. We used Fisher's exact test to evaluate the significance of differences between the allele frequencies in the two HD groups, between those in the two GD groups, and between those in each patient group and the control group. Data were analysed with JMP6 software (SAS Institute, Inc., Tokyo, Japan). A probability (P) values of less than 0·05 were considered significant.

Results

Statistical analysis revealed a significant association between the +869T/C polymorphism in the TGF-β1 gene and the

Table 1. Genotype and allele frequencies of the +869T/C polymorphism in patients with Hashimoto's disease (HD), in patients with Graves' disease (GD), and in control subjects.

	HD				GD				
	Severe ($n = 33$)	Mild ($n = 30$)	*P*-value	Odds ratio (95% CI)	Intractable ($n = 48$)	In remission ($n = 20$)	*P*-value	Odds ratio (95% CI)	Control ($n = 45$)
Genotype									
CC	6 (18·2%)	10 (33·3%)	0·0104*		13 (27·1%)	1 (5·0%)	0·0457*		11 (24·4%)
CT	13 (39·4%)	17 (56·7%)			19 (39·6%)	13 (65·0%)			21 (46·7%)
TT	14 (42·4%)	3 (10·0%)			16 (33·3%)	6 (30·0%)			13 (28·9%)
Allele									
C	25 (37·9%)	37 (61·7%)	0·0063†	2·6 (1·2–5·4)	45 (46·9%)	15 (37·5%)	n.s.†	1·5 (0·7–3·1)	43 (47·8%)
T	41 (62·1%)	23 (38·3%)			51 (53·1%)	25 (62·5%)			47 (52·2%)

*Value calculated by the χ^2 test. †Value calculated by Fisher's exact test. *†The *P*-values were calculated in comparisons within the disease group. CI, confidence interval; n.s., not significant.

pathological differences of HD and GD (Table 1). The frequency of the T allele was higher in patients with severe HD than in patients with mild HD (Table 1). The frequency of the TT genotype was higher in patients with severe HD than in patients with mild HD (Table 1).However, serum titres of McAb and TgAb did not differ significantly among the three groups with respect to the genotype (data not shown).

In GD patients, the frequency of the CC genotype was significantly higher in patients with intractable GD than in patients with GD in remission (Table 1), although there was no significant difference in the frequency of the C allele between patients with intractable GD and those with GD in remission (Table 1). Serum levels of TRAb did not differ significantly among the three groups with respect to genotype (data not shown).

Discussion

In the present study, we examined allele and genotypes frequencies of the +869T/C functional polymorphism in the TGF-β1 gene in Japanese patients with AITD and found that this polymorphism is associated with the occurrence of hypothyroidism in HD patients and disease intractability in GD. The frequency of the +869T allele was significantly higher in patients with severe HD than in those with mild HD (Table 1), and the frequency of the TT genotype was significantly lower in patients with mild HD than in those with severe HD (Table 1). *In vitro* transfection experiments that examined the change in TGF-β1 secretion by substitution of proline for leucine at 10 amino acid position in the signal peptide sequence of TGF-β1 [11] revealed that proline (+869C allele) shows 2·8-fold higer secretion of TGF-β1 than does leucine (+869T allele). Furthermore, the serum concentration of TGF-β1 is higher in individuals with the CC genotype than in those with the TT or TC genotype [12]. TGF-β1 suppresses the actions of cytotoxic T cells and expressions of IFN-γ [6], which are thought to be the main effector for thyroid destruction in HD [1–3]. Consistent with this, the proportion of activated cytotoxic T cells is lower in patients with mild HD than in those with severe HD [3]. In addition to this, previous reports show that IFN-γ levels are decreased in patients with mild HD [18] and that intracellular expression of IFN-γ in T cells is decreased in patients with mild HD [19]. Therefore, increased TGF-β1 may suppress T cell cytotoxicity in the thyroid and protect against the progression of hypothyroidism in HD patients with the +869C allele. In HD patients with the TT genotype, which is associated with low secretion of TGF-β1, T cell cytotoxicity against thyroid tissue may not be suppressed, resulting in the rapid progression of hypothyroidism. This hypothesis is supported by findings that systemic or local administration of TGF-β1 suppresses several autoimmune diseases associated with cell-mediated immune responses [20,21].

Recently, it has been reported that TGF-β1 converts $CD4^+$ $CD25^-$ T cells to $CD4^+$ $CD25^+$ forkhead box $P3^+$ ($FoxP3^+$) regulatory T (T_{reg}) cells *in vitro* [22,23]. TGF-β1 deficiency inhibits the suppressive function of T_{reg}, resulting in autoimmunity [24]. Over-expression of TGF-β1 in pancreatic islets expands the T_{reg} population and protects non-obese diabetic mice against type 1 diabetes [25]. In HD patients with the TGF-β1 +869C allele, the minimizing regulatory function of T_{reg} cells may be high autoimmune destruction of the thyroid gland.

In GD patients the frequency of the CC genotype was significantly higher in patients with intractable GD than in those with GD in remission (Table 1). Therefore, in contrast to HD, TGF-β1 may increase disease intractability or severity in GD. The reason why the effect of TGF-β1 on disease severity is different between HD and GD is unclear, but this may be due to the different aggravation mechanisms between HD and GD. Cell-mediated cytotoxicity exacerbates HD and antibody aggravates GD. Interestingly, there are many reports that TGF-β1 suppresses progression of autoimmune diseases where cell-mediated cytotoxicity plays an important role in tissue destruction, such as rheumatoid arthritis [21,26] and autoimmune insulitis [25,27]. In contrast, in autoimmune diseases where autoantibodies play an important role in the pathology, such as myasthenia gravis

[28,29], TGF-β1 aggravates the disease. In patients with asthma, which is caused by immunoglobulin E (IgE) antibody, TGF-β1 increases the disease severity [30]. Therefore, TGF-β1 may aggravate pathological conditions in particular immune diseases that are caused by antibodies such as autoantibodies and IgE.

In conclusion, the +869T/C polymorphism in the TGF-β1 gene is related to severity of HD and the intractability of GD patients.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture Japan. We would like to thank Y. Asami, S. Kuroda, T. Santo and M. Sugano for assistance with the collection of blood samples.

References

1 Volpe R. The immune system and its role in endocrine function. In: Becker KLS, ed. Principles and practice of endocrinology and metabolism. Philadelphia, PA: Lippincott Williams & Wilkins, 2001:1770–81.
2 Davies TF. Pathogenesis of Graves' disease. In: Braverman LE, Utiger RDS, eds. The thyroid: a fundamental and clinical text. Philadelphia, PA: Lippincott Williams & Wilkins, 2000:518–31.
3 Watanabe M, Yamamoto N, Maruoka H *et al.* Independent involvement of $CD8^+CD25^+$ cells and autoantibodies in disease severity of Hashimoto's disease. Thyroid 2002; **12**:801–8.
4 Ito C, Watanabe M, Okuda N *et al.* Association between the severity of Hashimoto's disease and the functional +874A/T polymorphism in the interferon-γ gene. Endocr J 2006; **53**:473–8.
5 Iwatani Y, Watanabe M. Normal mechanisms for self-tolerance. In: Volpe R, ed. Autoimmune endocrinopathies. Totawa, NJ: Humana Press, Inc., 1999:1–30.
6 Li MO, Wan YY, Sanjabi S *et al.* Transforming growth factor-β regulation of immune responses. Annu Rev Immunol 2006; **24**:99–146.
7 Derynck R, Jarrett JA, Chen EY *et al.* Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature 1985; **316**:701–5.
8 Lee G, Ellingsworth LR, Gillis S *et al.* Beta transforming growth factors are potential regulators of B lymphopoiesis. J Exp Med 1987; **166**:1290–9.
9 Cerwenka A, Swain SL. TGF-β1: immunosuppressant and viability factor for T lymphocytes. Microbes Infect 1999; **1**:1291–6.
10 Czarniecki CW, Chiu HH, Wong GH *et al.* Transforming growth factor-β1 modulates the expression of class II histocompatibility antigens on human cells. J Immunol 1988; **140**:4217–23.
11 Dunning AM, Ellis PD, McBride S *et al.* A transforming growth factor beta1 signal peptide variant increases secretion *in vitro* and is associated with increased incidence of invasive breast cancer. Cancer Res 2003; **63**:2610–15.
12 Yokota M, Ichihara S, Lin TL *et al.* Association of a T29→C polymorphism of the transforming growth factor-β1 gene with genetic susceptibility to myocardial infarction in Japanese. Circulation 2000; **101**:2783–7.
13 Sugiura Y, Niimi T, Sato S *et al.* Transforming growth factor β1 gene polymorphism in rheumatoid arthritis. Ann Rheum Dis 2002; **61**:826–8.
14 Lee EB, Kim JY, Lee YJ *et al.* Transforming growth factor-β1 polymorphisms in Korean patients with systemic sclerosis. Tissue Antigens 2004; **63**:491–5.
15 Hunt PJ, Marshall SE, Weetman AP *et al.* Cytokine gene polymorphisms in autoimmune thyroid disease. J Clin Endocrinol Metab 2000; **85**:1984–8.
16 Mysliwiec J, Kretowski A, Stepien A *et al.* Interleukin 18 and transforming growth factor β1 in the serum of patients with Graves' ophthalmopathy treated with corticosteroids. Int Immunopharmacol 2003; **3**:549–52.
17 Weetman AP. Chronic autoimmune thyroiditis. In: Braverman LE, Utiger RDS, eds. The thyroid. Philadelphia, PA: Lippincott Williams & Wilkins, 2000:721–32.
18 Roura-Mir C, Catalfamo M, Sospedra M *et al.* Single-cell analysis of intrathyroidal lymphocytes shows differential cytokine expression in Hashimoto's and Graves' disease. Eur J Immunol 1997; **27**:3290–302.
19 Mazziotti G, Sorvillo F, Naclerio C *et al.* Type-1 response in peripheral $CD4^+$ and $CD8^+$ T cells from patients with Hashimoto's thyroiditis. Eur J Endocrinol 2003; **148**:383–8.
20 Giladi E, Raz E, Karmeli F *et al.* Transforming growth factor-β gene therapy ameliorates experimental colitis in rats. Eur J Gastroenterol Hepatol 1995; **7**:341–7.
21 Thorbecke GJ, Shah R, Leu CH *et al.* Involvement of endogenous tumor necrosis factor alpha and transforming growth factor β during induction of collagen type II arthritis in mice. Proc Natl Acad Sci USA 1992; **89**:7375–9.
22 Chen W, Jin W, Hardegen N *et al.* Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003; **198**:1875–86.
23 Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci USA 2005; **102**:5126–31.
24 Aoki CA, Borchers AT, Li M, Flavell RA *et al.* Transforming growth factorβ (TGF-β) and autoimmunity. Autoimmun Rev 2005; **4**:450–9.
25 Peng Y, Laouar Y, Li MO, Green EA. TGF-beta regulates in vivo expansion of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells responsible for protection against diabetes. Proc Natl Acad Sci USA 2004; **101**:4572–7.
26 Brandes ME, Allen JB, Ogawa Y, Wahl SM. Transforming growth factor β1 suppresses acute and chronic arthritis in experimental animals. J Clin Invest 1991; **87**:1108–13.
27 Moritani M, Yoshimoto K, Wong SF *et al.* Abrogation of autoimmune diabetes in nonobese diabetic mice and protection against effector lymphocytes by transgenic paracrine TGF-β1. J Clin Invest 1998; **102**:499–506.
28 Xiao BG, Zhang GX, Shi FD *et al.* Decrease of LFA-1 is associated with upregulation of TGF-β in $CD4^+$ T cell clones derived from rats nasally tolerized against experimental autoimmune myasthenia gravis. Clin Immunol Immunopathol 1998; **89**:196–204.
29 Shi FD, Bai XF, Xiao BG *et al.* Nasal administration of multiple antigens suppresses experimental autoimmune myasthenia gravis, encephalomyelitis and neuritis. J Neurol Sci 1998; **155**:1–12.
30 Duvernelle C, Freund V, Frossard N. Transforming growth factor-β and its role in asthma. Pulm Pharmacol Ther 2003; **16**:181–96.

