Gene polymorphisms of *VEGF* and *VEGFR2* are associated with the severity of Hashimoto's disease and the intractability of Graves' disease, respectively

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Abstract. Vascular endothelial growth factor (VEGF) is one of main regulators of angiogenesis that functions by binding to its receptors, including VEGF receptor (VEGFR) 2. There are few data available regarding the association between *VEGF* and *VEGFR* polymorphisms and the susceptibility to and prognosis of autoimmune thyroid diseases (AITDs). To elucidate this association, we genotyped four functional *VEGF* and two *VEGFR2* polymorphisms and measured serum VEGF levels. In the four functional *VEGF* polymorphisms, the frequencies of the I carrier and I allele of *VEGF* -2549 I/D, which has lower activity, were higher in patients with severe HD than in those with mild HD. In the two functional *VEGFR2* polymorphisms, the frequency of the rs2071559 CC genotype, which has higher activity, was higher in patients with intractable GD than in controls, and the proportion of GD patients with larger goiters was higher in those with the CC genotype. Moreover, the frequency of the rs1870377 TT genotype with higher activity was higher in patients with intractable GD than in those with GD in remission. Combinations of *VEGF* and *VEGFR2* polymorphisms with stronger interactions were associated with the intractability of GD. Serum VEGF levels were higher in HD and AITD patients than those in controls. In conclusion, *VEGF* polymorphisms with lower activity were associated with the severity of HD, while *VEGFR2* polymorphisms and the combinations of *VEGF* and *VEGFR2* polymorphisms, which have stronger interactions, were associated with the intractability of GD. *VEGF* and *VEGFR2* polymorphisms were associated with HD severity and GD intractability, respectively.

Key words: Vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), Polymorphism, Severity, Intractability

AUTOIMMUNE THYROID DISEASES (AITDs), such as Graves' disease (GD) and Hashimoto's disease

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Abbreviations: AITDs, autoimmune thyroid diseases; GD, Graves' disease; HD, Hashimoto's disease; IFN, interferon; IL, interleukin; VEGFA, Vascular endothelial growth factor A; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; VEGFR, Vascular endothelial growth factor receptor; KD, Kawasaki disease; PBMC, peripheral blood mononuclear cells; FT4, free T4; FT3, free T3; TSH, thyrotropin; TRAb, anti-thyrotropin receptor antibody; TgAb, anti-thyroglobulin antibody; McAb, anti-thyroid microsomal antibody; EDTA, ethylenediamine tetraacetic acid; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ELISA, enzyme-linked immunosorbent assay; CTL, cytotoxic T lymphocytes; Th, helper T; sVEGFR2, soluble forms of VEGFR

(HD), are organ-specific autoimmune diseases [1, 2]. Their prevalences are reported to be relatively high at approximately 10% in various regions around the world including Japan [3]. Autoimmune diseases, including AITDs, are believed to develop by a combination of genetic and environmental factors [4, 5]. In twin studies, it was reported that the influence of genetic factors on AITD development is approximately 75% and that of environmental factors is approximately 25% [4]. The HLA-DR, CTLA-4, CD40, and TSHR genes were major susceptibility genes for AITD [6], and some environmental factors such as iodine and medications were reported [6].

The intractability of GD and the severity of HD are different among individuals. Some patients with GD achieve remission after treatment with anti-thyroid drugs, whereas others do not. Further, some patients with HD develop hypothyroidism in early life, while some maintain a euthyroid state. However, the intractability of GD and the severity of HD are very difficult to predict at

diagnosis. We have already reported that genetically higher expressions of Th1 cytokines and/or lower expressions of Th2 cytokines were associated with the severity of HD [7-9]. On the other hand, genetically higher expressions of Th17 cytokines were associated with intractability of GD [9-11].

In the thyroid gland of patients with GD, since both hypervascularity and increased blood flow are observed, angiogenesis is thought to be related to the development of goiter [12-15]. In addition, it is well known that it is difficult to achieve remission through medical treatment in GD patients with large goiters [16-22], and goiter size is thus considered important to predict the intractability of GD. In this study, we therefore focused on angiogenesis-related genes as one factor affecting goiter size.

Vascular endothelial growth factor A (VEGFA) is a key regulator of angiogenesis and is commonly called VEGF [23, 24]. VEGF induces the proliferation, migration, and survival of endothelial cells and also enhances vascular permeability [23, 24]. VEGF is expressed in many tissues and cells, including endothelial cells [25], macrophages, and some types of tumor cells [26]. In various cancer types, serum VEGF levels are higher relative to controls and show a positive correlation with cancer progression and patient survival [27, 28]. Moreover, serum VEGF levels are high and correlated with disease activity in various autoimmune diseases such as rheumatoid arthritis (RA), psoriasis vulgaris, and systemic lupus erythematosus (SLE) [29-32]. In the case of GD, increased serum VEGF levels can be found in treated euthyroid GD patients with larger goiters as well as untreated hyperthyroid GD patients [15]. In HD, serum VEGF levels are also higher in untreated hypothyroid HD patients relative to controls [15], whereas there is no difference in serum VEGF levels between treated euthyroid patients and controls [15]. On the other hand, another report showed that plasma VEGF levels are decreased in treated euthyroid HD patients compared to controls [33]. Taken together, these studies indicate that VEGF may be associated with the pathology or clinical condition of GD and HD.

Vascular endothelial growth factor receptor (VEGFR) mainly consists of VEGFR1 (fms-like tyrosine kinase-1 (Flt-1)), VEGFR2 (kinase insert domain receptor (KDR)/fetal liver kinase-1 (Flk-1)) and VEGFR3 (Flt-4) [34]. VEGF binds to VEGFR1 and VEGFR2 and activates the signaling pathways for angiogenesis [23, 24]. VEGFR2 plays a major role in angiogenesis [35, 36], regulates tumor angiogenesis [37, 38], and its overexpression is found in many types of tumors [39, 40].

Some gene polymorphisms are functional and can affect the expression or structure of a protein [41, 42]. In

the *VEGF* and *VEGFR2* genes, we focused on four functional *VEGF* polymorphisms (2549 I/D, rs1570360 G/A, rs2010963 C/G, and rs3025039 C/T) and two functional *VEGFR2* polymorphisms (rs2071559 C/T and rs1870377 A/T), which may affect angiogenesis-related diseases [43-51].

The D allele of *VEGF* -2549 I/D (18 bp insertion/deletion) polymorphism is associated with susceptibility to Kawasaki disease (KD) [43] and has 1.95-fold higher transcription activity than the I allele [44]. The G allele of *VEGF* rs1570360 G/A polymorphism is associated with the risk of renal allograft rejection and higher VEGF production from peripheral blood mononuclear cells (PBMC) [45]. The C allele of *VEGF* rs2010963 C/G polymorphism is associated with susceptibility to GD [46]. This allele also enhances serum VEGF levels [47] and VEGF mRNA expression [48]. The T allele of *VEGF* rs3025039 C/T polymorphism is associated with low risk of breast cancer [49] and KD [43], and this allele carrier shows lower levels of plasma VEGF [49].

In the case of the *VEGFR2* gene, the CC genotype of 2071559 C/T polymorphism shows decreased serum VEGFR2 levels [50]. The rs1870377 A/T polymorphism changes the sequence of the amino acids from His (T allele) to Gln (A allele), and the T allele shows higher binding affinity of VEGF to VEGFR2 [50]. This allele is also associated with susceptibility to astrocytomas [51].

In this study, we examined these polymorphisms in the VEGF and VEGFR2 genes and compared them between disease (AITD, GD, and HD) and control and between the different prognostic groups (intractable GD and GD in remission; severe HD and mild HD) to elucidate the association with the development and prognosis of AITD, respectively.

Material and Methods

Thyroid function and autoantibodies

Serum concentrations of free T4 (FT4), free T3 (FT3), and thyrotropin (TSH) were measured using ECLIA kit (Roche Diagnostics Ltd., Tokyo, Japan). Normal ranges of serum FT4, FT3, and TSH are 0.9–1.7 ng/dL, 2.3–4.3 pg/mL, and 0.5–5.0 μ IU/mL, respectively. TgAb and McAb were measured using a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titer >1:100 was considered positive. Serum TRAb at onset was measured by radioreceptor assay using a commercial kit (Cosmic Corp., Tokyo, Japan) as part of routine studies. Serum TRAb at the time of sampling was determined by ECLIA kit (third-generation) (Roche Diagnostics Ltd., Tokyo, Japan) using preserved samples. Normal value of TRAb was less than 10% in the radioreceptor assay and 2.0 IU/L in ECLIA. Goiter size in this study was defined

Table 1 Clinical characteristics of the subjects at the time of sampling in this study

	Controls	GD		HD	
		Past clinical history of thyrotoxicosis with elevated TRAb		Diffuse goiter and positive TgAb and/or McAb	
		Intractable	In remission	Severe	Mild
<i>n</i> (female/male)	117 (80/37)	97 (83/14)	58 (51/7)	118 (97/21)	81 (67/14)
The age of onset (years)		34.4 ± 14.0	33.6 ± 14.6	38.5 ± 9.9	58.8 ± 12.2
Age of the time of sampling (years)	33.7 ± 14.6	49.0 ± 15.0	44.8 ± 15.3	49.1 ± 14.1	62.7 ± 10.0
Goiter size (cm)	ND	4.9 ± 1.5	4.2 ± 0.6	4.1 ± 1.0*	4.6 ± 1.3
FT4 (ng/dL)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.2	1.3 ± 0.3**	1.2 ± 0.2
TSH (μIU/mL)	1.6 ± 1.0	1.7 ± 1.4	1.8 ± 1.2	4.1 ± 10.3	2.8 ± 1.9
TRAb (IU/L)	<2.0	13.6 ± 60.1	<2.0	<2.0	<2.0
TgAb (2 ⁿ × 100) ^a	Negative	2.8 ± 3.0	2.9 ± 3.2	6.6 ± 3.2***	1.3 ± 2.4
McAb (2 ⁿ × 100) ^a	Negative	4.4 ± 2.7	5.5 ± 2.0	5.4 ± 3.0	3.5 ± 2.8
Current treatment	None	MMI or PTU	None	L-thyroxine	None
Current dose of anti-thyroid drug (mg/day) ^b (range)	None	14.5 ± 20.8 (2.5–150)	None	None	None
Current dose of L-thyroxine (μg/day) (range)	None	None	None	80.0 ± 37.2 (12.5–250)	None

Data are expressed as mean ± standard deviation

ND, not determined; PTU, propylthiouracil; MMI, methimazole

^a When the titer of TgAb or McAb was 25,600, it was expressed as 2⁸ × 100, ^b Doses were expressed as the comparable dose of MMI (50 mg of PTU was converted to 5 mg of MMI)

Analyzed by Mann-Whitney *U* test, * *p* = 0.0295 (vs. mild HD), ** *p* = 0.0001 (vs. mild HD), *** *p* = 0.0009 (vs. mild HD)

as the longitudinal length of the thyroid gland as measured using a vernier caliper.

Subjects for genotyping

We genotyped each polymorphism in 462 AITD patients and 117 healthy controls. Among the 214 GD patients who had a clinical history of thyrotoxicosis and were positive for anti-thyrotropin receptor antibody (TRAb), 97 patients who had been treated with methimazole for at least 5 years were still positive for TRAb (intractable GD), 58 patients with GD who had maintained a euthyroid state were negative for TRAb for more than 2 years without medication (GD in remission), and 59 patients could not be classified into either intractable GD or GD in remission groups at the time of analysis.

Among 248 HD patients who were positive for anti-thyroglobulin antibody (TgAb) and/or anti-microsomal antibody (McAb), we genotyped 118 patients who developed moderate to severe hypothyroidism before 50 years of age and were treated with thyroxine (severe HD), 81 untreated euthyroid patients who were over 50 years of age (mild HD), and 49 patients who could not be classified into either severe HD or mild HD groups at the time of analysis. Healthy controls were euthyroid and nega-

tive for any thyroid autoantibodies. Table 1 shows the clinical characteristics of all subjects in this study. Goiter size was larger in patients with mild HD than that in patients with severe HD (*p* = 0.0295). FT4 levels were higher in patients with severe HD than those in patients with mild HD (*p* = 0.0001). The titers of TgAb were significantly higher in patients with severe HD than those in patients with mild HD (*p* = 0.0004), as per our previous report [52].

Genomic DNA was isolated from ethylenediamine tetraacetic acid (EDTA)-treated whole blood cells with a commercially available kit (QIAamp® DNA Blood Mini Kit, QIAGEN, Tokyo, Japan) according to manufacture's protocol. Briefly, 200 μL blood sample and 20 μL protease were added into a 1.5 mL microcentrifuge tube. These samples were then incubated at 56°C for 10 min and centrifuged. We pipetted these mixtures onto the QIAamp Mini spin column and centrifuged them at 6,000 × *g* (8,000 rpm) for 1 min. Finally, we eluted the DNA in a new 1.5 mL microcentrifuge tube by centrifuging them at 6,000 × *g* (8,000 rpm) for 1 min. Written informed consent was obtained from all patients and control subjects, and the study protocol was approved by the Ethics Committee of Osaka University (564).

Genotyping of polymorphisms

Genotyped polymorphisms in this study are shown in Table 2. *VEGF* -2549 I/D polymorphism was analyzed using the polymerase chain reaction (PCR) method. The other polymorphisms (*VEGF* rs1570360 A/G, rs2010963 C/G and rs3025039, and *VEGFR2* rs2071559 C/T and rs1870377 A/T) were analyzed by the polymerase chain reaction-restriction fragment length polymorphism

(PCR-RFLP) method. The target sequences of each gene were amplified using PCR, and the PCR products were digested by the addition of restriction enzymes. The primers, the PCR conditions, restriction enzymes used in this study, and electrophoresis image obtained are shown in Table 3 and Fig. 1.

Table 2 Functions of *VEGF* and *VEGFR2* gene polymorphisms targeted in this study

Gene	Polymorphism	Region	Effect to expression
<i>VEGF</i>	-2549 I/D	promoter	D allele > I allele (transcriptional activity)
	rs1570360	promoter	GG genotype > AA genotype (production levels)
	rs2010963	5'-UTR	CC genotype > GG genotype (serum levels and expression levels)
	rs3025039	3'-UTR	CC genotype > T carrier (plasma levels)
<i>VEGFR2</i>	rs2071559	promoter	CC genotype < TT genotype (serum soluble VEGFR2 levels)
	rs1870377	exon 11	T allele > A allele (binding efficiency)

Fig. 1 Representative electrophoresis image to genotype *VEGF* and *VEGFR* polymorphisms in this study.

(A) *VEGF* -2549 I/D, (B) *VEGF* rs1570360, (C) *VEGF* rs2010963, (D) *VEGF* rs3025039, (E) *VEGFR* rs2071559, and (F) *VEGFR* rs1870377 polymorphisms. Detailed information on each genotype is shown in Table 3.

Table 3 Primers, PCR conditions, and methods (restriction enzymes) used in this study

Gene	Polymorphism	PCR primer	PCR conditions	Methods (Restriction enzymes)	PCR products or Restriction fragments
<i>VEGF</i>	-2549 I/D	5'-GGCCTTAGGACACCATAACCG-3' 5'-AGGGAACAAAGTTGGGGCTC-3'	95°C for 5 min (95°C for 30 sec, 60.2°C for 30 sec, 72°C for 20 sec) × 29 cycle 72°C for 5 min	PCR	II genotype: 296 bp ID genotype: 296 bp + 278 bp DD genotype: 278 bp
	rs1570360	5'-TCCTGCTCCCTCCTCGCCAATG-3' 5'-GGCGGGGACAGGCGAGCATC-3'	95°C for 5 min (95°C for 30 sec, 61.1°C for 30 sec, 72°C for 30 sec) × 35 cycle 72°C for 5 min	PCR-RFLP (MnII)	AA genotype: 206 bp AG genotype: 206 bp + 187 bp + 19 bp GG genotype: 187 bp + 19 bp
	rs2010963	5'-GGAGATTGCTCTACTTCCCCAA-3' 5'-GTCTGTCTGTCTGTCCGTCA-3'	95°C for 5 min (95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec) × 30 cycle 72°C for 5 min	PCR-RFLP (BsmFI)	CC genotype: 256 bp GC genotype: 256 bp + 145 bp + 111 bp GG genotype: 145 bp + 111 bp
	rs3025039	5'-GATACAGAAACCACGCTGCC-3' 5'-CGGGCTCGGTGATTAGCAG-3'	95°C for 5 min (95°C for 30 sec, 60.6°C for 30 sec, 72°C for 30 sec) × 35 cycle 72°C for 5 min	PCR-RFLP (NlaIII)	CC genotype: 219 bp CT genotype: 219 bp + 160 bp + 59 bp TT genotype: 160 bp + 59 bp
<i>VEGFR2</i>	rs2071559	5'-CAAACCTTCACTAGGGCTCTTCGT-3' 5'-AGCCACAAGGGAGAAGCGGATA-3'	95°C for 5 min (95°C for 30 sec, 61°C for 30 sec, 72°C for 30 sec) × 30 cycle 72°C for 5 min	PCR-RFLP (BsmI)	TT genotype: 290 bp CT genotype: 290 bp + 174 bp + 116 bp CC genotype: 174 bp + 116 bp
	rs1870377	5'-GCCTCACATATTATTGTACCATCC-3' 5'-CCTCCTGTATCCTGAATGAATCT-3'	95°C for 5 min (95°C for 30 sec, 60.5°C for 30 sec, 72°C for 30 sec) × 29 cycle 72°C for 5 min	PCR-RFLP (AluI)	AA genotype: 404 bp AT genotype: 404 bp + 213 bp + 191 bp TT genotype: 191 bp + 213 bp

Serum VEGF levels

We also selected age-matched subjects for measurement of serum VEGF levels. Serum VEGF levels were examined in 32 GD patients (16 intractable GD patients and 16 GD patients in remission), 32 HD patients (16 severe HD patients and 16 mild HD patients), and 14 healthy controls. We examined the difference in serum VEGF levels in each genotype of *VEGF* polymorphism by using healthy controls, untreated GD patients in remission, and untreated mild HD patients to avoid the effect of medical treatment. Serum VEGF levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine Human VEGF Immunoassay, R&D Systems, Inc., Minneapolis,

MN, USA). Table 4 shows the clinical characteristics of all subjects used in this analysis. Goiter size was larger in intractable GD patients than that in GD patients in remission ($p = 0.0004$), as well as in mild HD patients relative to severe HD patients ($p = 0.0052$). FT4 levels were higher in patients with severe HD than those in patients with mid HD ($p = 0.0012$).

Statistical analysis

We used the χ^2 test and Fisher's exact test to evaluate the significance of the differences in the frequencies of genotypes and alleles and goiter size among the groups. The Mann-Whitney U test was used to analyze the differences between the titers of McAb and TgAb and the

Table 4 Clinical characteristics of the subjects obtained by analyzing the serum VEGF levels

	Controls	GD		HD	
		Past clinical history of thyrotoxicosis with elevated TRAb		Diffuse goiter and positive TgAb and/or McAb	
		Intractable	In remission	Severe	Mild
<i>n</i> (female/male)	14 (11/3)	16 (14/2)	16 (14/2)	16 (14/2)	16 (14/2)
The age of onset (years)		34.4 ± 15.2	35.4 ± 13.7	38.3 ± 9.9	54.9 ± 6.1
Age at the time of sampling (years)	51.1 ± 16.7	54.1 ± 11.9	54.5 ± 10.7	59.1 ± 14.2	60.9 ± 6.1
Goiter size (cm)	ND	5.0 ± 1.0*	3.8 ± 0.4	3.6 ± 0.5**	4.2 ± 0.7
Free T4 (ng/dL)	1.2 ± 0.3	1.1 ± 0.1	1.2 ± 0.2	1.3 ± 0.1***	1.1 ± 0.2
TSH (μU/mL)	1.6 ± 1.0	1.9 ± 1.1	1.6 ± 0.6	2.0 ± 0.8	2.2 ± 1.1
TRAb (IU/L)	<2.0	13.9 ± 22.4	<2.0	<2.0	<2.0
Current treatment	None	MMI or PTU	None	L-thyroxine	None
Current dose of anti-thyroid drug (mg/day) ^a (range)	None	14.1 ± 11.8 (1.25–30)	None	None	None
Current dose of L-thyroxine (μg/day) (range)	None	None	None	84.3 ± 31.1 (37.5–150)	None

Data are expressed as mean ± standard deviation

ND, not determined; PTU, propylthiouracil; MMI, methimazole

^a Doses were expressed as the comparable dose of MMI (50 mg of PTU was converted to 5 mg of MMI)

Analyzed by Mann-Whitney *U* test, * *p* = 0.0004 (vs. GD in remission), ** *p* = 0.0052 (vs. mild HD), ****p* = 0.0026 (vs. mild HD)

levels of TRAb among each genotype. Student's *t* test, the Mann-Whitney *U* test, and the χ^2 test were used to analyze differences in serum VEGFA levels. The data were analyzed with JMP 13 software (SAS Institute Inc., Tokyo, Japan). Probability values of <0.05 were considered significant. The sample number in this study was required to detect approximately 20% difference in *VEGF* −2549DD genotype frequency with 80% power (α = 0.05).

Results

VEGF polymorphisms

We could not find any significant differences in the genotype and allele frequencies of each examined polymorphism in *VEGF* genes between each patient group and control subjects (Table 5). However, the frequencies of the I carrier (II + ID genotypes) and the I allele of the functional *VEGF* −2549 I/D polymorphism were higher in patients with severe HD than in those with mild HD (*p* = 0.0430 and 0.0338, respectively) (Table 6). In the *VEGF* rs3025039 polymorphism, the proportion of patients with larger goiters (>5.6 cm) was higher in GD patients with the CC genotype than in T carriers (*p* = 0.0239) (Fig. 2).

VEGFR2 polymorphisms

We could not find any significant differences in the genotype and allele frequencies of each examined polymorphism in the *VEGFR2* gene between each patient group and control subjects (Table 7). However, the frequency of the CC genotype of *VEGFR2* rs2071559 was higher in patients with intractable GD than that in control subjects (*p* = 0.0362) (Table 8). The proportion of GD patients with larger goiters (>5.6 cm) was higher in subjects with CC genotype than those with T carriers (Fig. 3). The frequency of the TT genotype of the *VEGFR2* rs1870377 polymorphism was higher in intractable GD than GD in remission (*p* = 0.0438) (Table 8).

Combined analysis of *VEGF* and *VEGFR2* polymorphisms

We performed combined analysis of the polymorphisms in the *VEGF* and *VEGFR2* genes between intractable GD patients and patients in remission. The frequencies of the GG/CC genotypes of *VEGF* rs1570360/*VEGFR2* rs2071559 polymorphisms, C carrier/CC genotype of *VEGF* rs2010963/*VEGFR2* rs2071559 polymorphisms, C carrier/CC genotype of *VEGF* rs3025039/*VEGFR2* rs2071559 polymorphisms, D carrier/TT genotype of *VEGF* −2549 I/D/*VEGFR2* rs1870377 polymor-

phisms, and G carrier/TT genotype of *VEGF* rs1570360/*VEGFR2* rs1870377 polymorphisms were higher in patients with intractable GD than that in patients with GD in remission ($p = 0.0427, 0.0222, 0.0491, 0.0326$, and 0.0342 , respectively) (Table 9).

Table 5 Genotype and allele frequencies of the *VEGF* polymorphisms in patients with AITD and in control subjects

		Control	All patients with AITD		All patients with GD		All patients with HD	
<i>VEGF</i> -2549 I/D	II	11 (11.22%)	33 (7.91%)		15 (7.94%)		18 (7.9%)	
	ID	35 (35.72%)	157 (37.65%)	NS ^b	73 (38.62%)	NS ^b	84 (36.84%)	NS ^b
	DD	52 (53.06%)	227 (54.44%)		101 (53.44%)		126 (55.26%)	
	II + ID	46 (46.94%)	190 (45.56%)		88 (46.56%)		102 (44.74%)	
	DD	52 (53.06%)	227 (54.44%)	NS ^b	101 (53.44%)	NS ^b	126 (55.26%)	NS ^b
	II	11 (11.22%)	33 (7.91%)		15 (7.94%)		18 (7.9%)	
	ID + DD	87 (88.78%)	384 (92.09%)	NS ^b	174 (92.06%)	NS ^b	210 (92.1%)	NS ^b
	I allele	57 (29.08%)	223 (26.74%)	NS ^b	103 (27.25%)	NS ^b	120 (26.32%)	NS ^b
<i>VEGF</i> rs1570360	D allele	139 (70.92%)	611 (73.26%)		275 (72.75%)		336 (73.68%)	
	AA	3 (3%)	2 (0.49%)		1 (0.54%)		1 (0.45%)	
	AG	24 (24%)	88 (21.57%)	NS ^{a,b}	43 (23.37%)	NS ^{a,b}	45 (20.09%)	NS ^{a,b}
	GG	73 (73%)	318 (77.94%)		140 (76.09%)		178 (79.46%)	
	AA + AG	27 (27%)	90 (22.06%)		44 (23.91%)		46 (20.54%)	
	GG	73 (73%)	318 (77.94%)	NS ^b	140 (76.09%)	NS ^b	178 (79.46%)	NS ^b
	AA	3 (3%)	2 (0.49%)		1 (0.54%)		1 (0.45%)	
	AG + GG	97 (97%)	406 (99.51%)	NS ^{a,b}	183 (99.46%)	NS ^{a,b}	223 (99.55%)	NS ^{a,b}
<i>VEGF</i> rs2010963	A allele	30 (15%)	92 (11.27%)	NS ^b	45 (12.23%)	NS ^b	47 (10.49%)	NS ^b
	G allele	170 (85%)	724 (88.73%)		323 (87.77%)		401 (89.51%)	
	CC	18 (16.36%)	95 (22.78%)		43 (23.12%)		52 (22.51%)	
	GC	56 (50.91%)	202 (48.44%)	NS ^b	95 (51.07%)	NS ^b	107 (46.32%)	NS ^b
	GG	36 (32.73%)	120 (28.78%)		48 (25.81%)		72 (31.17%)	
	CC + GC	74 (67.27%)	297 (71.22%)		138 (74.19%)		159 (68.83%)	
	GG	36 (32.73%)	120 (28.78%)	NS ^b	48 (25.81%)	NS ^b	72 (31.17%)	NS ^b
	CC	18 (16.36%)	95 (22.78%)		43 (23.12%)		52 (22.51%)	
<i>VEGF</i> rs3025039	GC + GG	92 (83.64%)	322 (77.22%)	NS ^b	143 (76.88%)	NS ^b	179 (77.49%)	NS ^b
	C allele	92 (41.82%)	392 (47%)	NS ^b	181 (48.66%)	NS ^b	211 (45.67%)	NS ^b
	G allele	128 (58.18%)	442 (53%)		191 (51.34%)		251 (54.33%)	
	CC	67 (65.69%)	275 (66.1%)		121 (64.02%)		154 (67.84%)	
	CT	29 (28.43%)	123 (29.57%)	NS ^b	58 (30.69%)	NS ^b	65 (28.64%)	NS ^b
	TT	6 (5.88%)	18 (4.33%)		10 (5.29%)		8 (3.52%)	
	CC + CT	96 (94.12%)	398 (95.67%)		179 (94.71%)		219 (96.48%)	
	TT	6 (5.88%)	18 (4.33%)	NS ^{a,b}	10 (5.29%)	NS ^b	8 (3.52%)	NS ^{a,b}
<i>VEGF</i> rs3025039	CC	67 (65.69%)	275 (66.1%)		121 (64.02%)		154 (67.84%)	
	CT + TT	35 (34.31%)	141 (33.9%)	NS ^b	68 (35.98%)	NS ^b	73 (32.16%)	NS ^b
	C allele	163 (79.9%)	673 (80.89%)	NS ^b	300 (79.37%)	NS ^b	373 (82.16%)	NS ^b
	T allele	41 (20.1%)	159 (19.11%)		78 (20.63%)		81 (17.84%)	

Analyzed by χ^2 test except ^a analyzed by Fisher's exact test. ^b vs. control, NS: not significant

Table 6 Genotype and allele frequencies of *VEGF* polymorphisms genotyped in this study in patients with HD and GD

		Control	GD			HD		
			Intractable	In remission		Severe	Mild	
<i>VEGF</i> −2549 I/D	II	11 (11.22%)	9 (9.89%)	3 (5.26%)	NS ^b	11 (9.82%)	4 (5.26%)	NS ^c
	ID	35 (35.72%)	38 (41.76%)	21 (36.84%)		44 (39.29%)	22 (28.95%)	
	DD	52 (53.06%)	44 (48.35%)	33 (57.9%)		57 (50.89%)	50 (65.79%)	
	II + ID	46 (46.94%)	47 (51.65%)	24 (42.1%)	NS ^b	55 (49.11%)	26 (34.21%)	0.0430 ^c
	DD	52 (53.06%)	44 (48.35%)	33 (57.9%)		57 (50.89%)	50 (65.79%)	
	II	11 (11.22%)	9 (9.89%)	3 (5.26%)	NS ^{a,b}	11 (9.82%)	4 (5.26%)	NS ^c
	ID + DD	87 (88.78%)	82 (90.11%)	54 (94.74%)		101 (90.18%)	72 (94.74%)	
	I allele	57 (29.08%)	56 (30.77%)	27 (23.68%)	NS ^b	66 (29.46%)	30 (19.74%)	0.0338 ^c
	D allele	139 (70.92%)	126 (69.23%)	87 (76.32%)		158 (70.54%)	122 (80.26%)	
<i>VEGF</i> rs1570360	AA	3 (3%)	1 (1.12%)	0 (0%)	NS ^{a,b}	1 (0.9%)	0 (0%)	NS ^{a,c}
	AG	24 (24%)	23 (25.84%)	11 (20%)		26 (23.42%)	11 (15.07%)	
	GG	73 (73%)	65 (73.04%)	44 (80%)		84 (75.68%)	62 (84.93%)	
	AA + AG	27 (27%)	24 (26.96%)	11 (20%)	NS ^b	27 (24.32%)	11 (15.07%)	NS ^c
	GG	73 (73%)	65 (73.04%)	44 (80%)		84 (75.68%)	62 (84.93%)	
	AA	3 (3%)	1 (1.12%)	0 (0%)	NS ^{a,b}	1 (0.9%)	0 (0%)	NS ^{a,c}
	AG + GG	97 (97%)	88 (98.88%)	55 (100%)		110 (99.1%)	73 (100%)	
	A allele	30 (15%)	25 (14.04%)	11 (10%)	NS ^b	28 (12.61%)	11 (7.53%)	NS ^c
	G allele	170 (85%)	153 (85.96%)	99 (90%)		194 (87.39%)	135 (92.47%)	
<i>VEGF</i> rs2010963	CC	18 (16.36%)	19 (21.11%)	12 (21.43%)	NS ^b	22 (19.3%)	24 (31.17%)	NS ^c
	GC	56 (50.91%)	43 (47.78%)	31 (55.36%)		54 (47.37%)	29 (37.66%)	
	GG	36 (32.73%)	28 (31.11%)	13 (23.21%)		38 (33.33%)	24 (31.17%)	
	CC + GC	74 (67.27%)	62 (68.89%)	43 (76.79%)	NS ^b	76 (66.67%)	53 (68.83%)	NS ^c
	GG	36 (32.73%)	28 (31.11%)	13 (23.21%)		38 (33.33%)	24 (31.17%)	
	CC	18 (16.36%)	19 (21.11%)	12 (21.43%)	NS ^b	22 (19.3%)	24 (31.17%)	NS ^c
	GC + GG	92 (83.64%)	71 (78.89%)	44 (78.57%)		92 (80.7%)	53 (68.83%)	
	C allele	92 (41.82%)	81 (45%)	55 (49.11%)	NS ^b	98 (42.98%)	77 (50%)	NS ^c
	G allele	128 (58.18%)	99 (55%)	57 (50.89%)		130 (57.02%)	77 (50%)	
<i>VEGF</i> rs3025039	CC	67 (65.69%)	55 (61.11%)	35 (60.34%)	NS ^{a,b}	76 (66.67%)	52 (71.23%)	NS ^{a,c}
	CT	29 (28.43%)	28 (31.11%)	22 (37.93%)		35 (30.7%)	19 (26.03%)	
	TT	6 (5.88%)	7 (7.78%)	1 (1.73%)		3 (2.63%)	2 (2.74%)	
	CC + CT	96 (94.12%)	83 (92.22%)	57 (98.27%)	NS ^{a,b}	111 (97.37%)	71 (97.26%)	NS ^{a,c}
	TT	6 (5.88%)	7 (7.78%)	1 (1.73%)		3 (2.63%)	2 (2.74%)	
	CC	67 (65.69%)	55 (61.11%)	35 (60.34%)	NS ^b	76 (66.67%)	52 (71.23%)	NS ^c
	CT + TT	35 (34.31%)	35 (38.89%)	23 (39.66%)		38 (33.33%)	21 (28.77%)	
	C allele	163 (79.9%)	138 (76.67%)	92 (79.31%)	NS ^b	187 (82.02%)	123 (84.25%)	NS ^c
	T allele	41 (20.1%)	42 (23.33%)	24 (20.69%)		41 (17.98%)	23 (15.75%)	

Analyzed by χ^2 test except ^a analyzed by Fisher's exact test. ^b intractable GD vs. GD in remission, ^c severe HD vs. mild HD, NS: not significant

Table 7 Genotype and allele frequencies of the *VEGFR2* polymorphisms in patients with AITD and in control subjects

		Control	All patients with AITD		All patients with GD		All patients with HD	
<i>VEGFR2</i> rs2071559	CC	3 (2.89%)	28 (6.68%)		13 (6.99%)		15 (6.44%)	
	CT	35 (33.65%)	149 (35.56%)	NS ^a	65 (34.95%)	NS ^a	84 (36.05%)	NS ^a
	TT	66 (63.46%)	242 (57.76%)		108 (58.06%)		134 (57.51%)	
	CC + CT	38 (36.54%)	177 (42.24%)	NS ^a	78 (41.94%)	NS ^a	99 (42.49%)	NS ^a
	TT	66 (63.46%)	242 (57.76%)		108 (58.06%)		134 (57.51%)	
	CC	3 (2.89%)	28 (6.68%)	NS ^a	13 (6.99%)	NS ^a	15 (6.44%)	NS ^a
	CT + TT	101 (97.11%)	391 (93.32%)		173 (93.01%)		218 (93.56%)	
	C allele	41 (19.71%)	205 (24.46%)	NS ^a	91 (24.46%)	NS ^a	114 (24.46%)	NS ^a
<i>VEGFR2</i> rs1870377	T allele	167 (80.29%)	633 (75.54%)		281 (75.54%)		352 (75.54%)	
	AA	12 (11.88%)	52 (12.59%)		24 (12.7%)		28 (12.5%)	
	AT	54 (53.47%)	220 (53.27%)	NS ^a	107 (56.61%)	NS ^a	113 (50.45%)	NS ^a
	TT	35 (34.65%)	141 (34.14%)		58 (30.69%)		83 (37.05%)	
	AA + AT	66 (65.35%)	272 (65.86%)	NS ^a	131 (69.31%)	NS ^a	141 (62.95%)	NS ^a
	TT	35 (34.65%)	141 (34.14%)		58 (30.69%)		83 (37.05%)	
	AA	12 (11.88%)	52 (12.59%)	NS ^a	24 (12.7%)	NS ^a	28 (12.5%)	NS ^a
	AT + TT	89 (88.12%)	361 (87.41%)		165 (87.3%)		196 (87.5%)	
	A allele	78 (38.61%)	324 (39.23%)	NS ^a	155 (41.01%)	NS ^a	169 (37.72%)	NS ^a
	T allele	124 (61.39%)	502 (60.77%)		223 (58.99%)		279 (62.28%)	

Analyzed by χ^2 test, ^a vs. control, NS: not significant

Fig. 2 Goiter size of GD patients in each genotype of *VEGF* rs3025039 polymorphism.

Size of a goiter that could not be measured by palpation was defined as 3.5 cm. Difference in the proportion of patients with larger goiter size (>5.6 cm) was analyzed by χ^2 test.

Fig. 3 Goiter size of GD patients in each genotype of *VEGFR2* rs2071559 polymorphism.

Size of a goiter that could not be measured by palpation was defined as 3.5 cm. Difference in the proportion of patients with larger goiter size (>5.6 cm) was analyzed by χ^2 test.

Serum VEGF levels

Serum VEGF levels were higher in HD and AITD patients than those in control subjects ($p = 0.0154$,

0.0208, respectively) (Fig. 4) and tended to be higher, but not significantly so, in GD patients than those in control subjects ($p = 0.0507$) (Fig. 4). However, there were

Table 8 Genotype and allele frequencies of *VEGFR2* polymorphisms genotyped in this study in patients with HD and GD

		Control	GD			HD		
			Intractable	In remission		Severe	Mild	
VEGFR2 rs2071559	CC	3 (2.89%)	9 (10.23%)	1 (1.75%)	NS ^b	8 (7.02%)	6 (8.11%)	NS ^c
	CT	35 (33.65%)	29 (32.95%)	18 (31.58%)		41 (35.96%)	24 (32.43%)	
	TT	66 (63.46%)	50 (56.82%)	38 (66.67%)		65 (57.02%)	44 (59.46%)	
	CC + CT	38 (36.54%)	38 (43.18%)	19 (33.33%)	NS ^b	49 (42.98%)	30 (40.54%)	NS ^c
	TT	66 (63.46%)	50 (56.82%)	38 (66.67%)		65 (57.02%)	44 (59.46%)	
	CC	3 (2.89%)	9 (10.23%)^d	1 (1.75%)	NS ^{a,b}	8 (7.02%)	6 (8.11%)	NS ^c
	CT + TT	101 (97.11%)	79 (89.77%)	56 (98.25%)		106 (92.98%)	68 (91.89%)	
	C allele	41 (19.71%)	47 (26.7%)	20 (17.54%)	NS ^b	57 (25%)	36 (24.32%)	NS ^c
	T allele	167 (80.29%)	129 (73.3%)	94 (82.46%)		171 (75%)	112 (75.68%)	
VEGFR2 rs1870377	AA	12 (11.88%)	10 (10.75%)	8 (14.03%)	NS ^b	11 (9.73%)	10 (14.08%)	NS ^c
	AT	54 (53.47%)	47 (50.54%)	36 (63.16%)		62 (54.87%)	33 (46.48%)	
	TT	35 (34.65%)	36 (38.71%)	13 (22.81%)		40 (35.4%)	28 (39.44%)	
	AA + AT	66 (65.35%)	57 (61.29%)	44 (77.19%)	0.0438 ^b	73 (64.6%)	43 (60.56%)	NS ^c
	TT	35 (34.65%)	36 (38.71%)	13 (22.81%)		40 (35.4%)	28 (39.44%)	
	AA	12 (11.88%)	10 (10.75%)	8 (14.03%)	NS ^b	11 (9.73%)	10 (14.08%)	NS ^c
	AT + TT	89 (88.12%)	83 (89.25%)	49 (85.97%)		102 (90.27%)	61 (85.92%)	
	A allele	78 (38.61%)	67 (36.02%)	52 (45.61%)	NS ^b	84 (37.17%)	53 (37.32%)	NS ^c
	T allele	124 (61.39%)	119 (63.98%)	62 (54.39%)		142 (62.83%)	89 (62.68%)	

Analyzed by χ^2 test except ^a analyzed by Fisher's exact test. ^b Intractable GD vs. GD in remission, ^c severe HD vs. mild HD, ^d $p = 0.0362$ (vs. control), NS: not significant

Table 9 Combined analysis to study the association between polymorphisms in *VEGF* and *VEGFR2* among GD

Genotype of gene polymorphisms		GD		
		Intractable	In remission	
<i>VEGF</i> rs1570360/ <i>VEGFR2</i> rs2071559	GG/CC others	7 (8.14%) 79 (91.86%)	0 (0%) 55 (100%)	0.0427 ^a
<i>VEGF</i> rs2010963/ <i>VEGFR2</i> rs2071559	CC + CG/CC others	8 (9.3%) 78 (90.7%)	0 (0%) 56 (100%)	0.0222 ^a
<i>VEGF</i> rs3025039/ <i>VEGFR2</i> rs2071559	CC + CT/CC others	9 (10.71%) 75 (89.29%)	1 (1.75%) 56 (98.25%)	0.0491 ^a
<i>VEGF</i> -2549 I/D/ <i>VEGFR2</i> rs1870377	DD + ID/TT others	33 (37.93%) 54 (62.07%)	12 (21.05%) 45 (78.95%)	0.0326
<i>VEGF</i> rs1570360/ <i>VEGFR2</i> rs1870377	GG + GA/TT others	36 (40.91%) 52 (59.09%)	13 (23.64%) 42 (76.36%)	0.0342

Analyzed by χ^2 test except ^a analyzed by Fisher's exact test.

no significant differences in serum VEGF levels between intractable GD patients and GD patients in remission or between severe HD patients and mild HD patients (data not shown).

To clarify the effect of VEGF polymorphism on serum VEGF levels, we analyzed the association between

serum VEGF levels and genotypes of VEGF polymorphism. The levels of serum VEGF were higher in the DD genotype of the *VEGF* -2549 I/D polymorphism than in I carriers (DI + II genotypes) (Fig. 5A), in the GG genotype of the *VEGF* rs1570360 polymorphism than in the GA genotype (Fig. 5B), in the CC genotype of *VEGF*

Fig. 4 Serum VEGF levels in AITD patients and controls. Analyzed by ^a Student's *t* test and ^b Mann-Whitney *U* test.

rs2010963 than that in G carriers (GG + GC genotypes) (Fig. 5C), and in the CC genotype of VEGF rs3025039 than that in T carriers (CT + TT genotypes) (Fig. 5D), but not significantly so.

However, we found that the proportion of subjects with higher serum VEGF levels (>325 pg/mL) was higher in the CC genotype of VEGF rs2010963 than that in G carriers (GG + GC genotypes) ($p = 0.0430$) (Fig. 5C) and that the proportion of subjects with higher serum VEGF levels (>300 pg/mL) was higher in the CC genotype of VEGF rs3025039 than that in T carriers (CT + TT genotypes) ($p = 0.0244$) (Fig. 5D).

Discussion

In this study, we expected that enhanced angiogenesis in thyroid glands of GD patients would be associated with larger goiters and intractability of GD. In HD, we also expected that enhanced angiogenesis would cause severe infiltration of immune cells, such as cytotoxic T lymphocytes (CTL) and helper T (Th) 1 cells, to the thyroid and lead to the destruction of the thyroid glands.

In VEGF -2549 I/D polymorphism, we hypothesized that the frequency of the D allele would be increased in severe HD patients relative to mild HD patients because this allele has higher transcriptional activity [44]. Contrary to our expectation, we found that the frequencies of the I carrier and I allele, which are associated with lower VEGF expression [44], were higher in severe HD patients relative to mild HD patients (Table 6). Previous reports have shown that the AA genotype of rs699947, which is linked to the II genotype of -2549 I/D [44, 53], is associated with susceptibility to and early onset of type 1 diabetes, suggesting that VEGF may play a protective role in diabetes [54]. Moreover, other reports

show that VEGF enhances survival of brown adipocytes [55], as well as endothelial cells [23, 24]. VEGF also has neuroprotective effects in mice with experimental autoimmune encephalomyelitis [56]. On the other hand, serum level of IFN- γ was significantly lower in a group of colorectal carcinoma patients with positive staining of VEGF [57]. We have already reported that genetically higher expressions of IFN- γ and high Th1/Th2 ratio were associated with the severity of HD [7, 9]. Therefore, we suggested that VEGF might protect thyrocytes from CTL and Th1 cells and suppress IFN- γ production in HD.

In the case of GD, a previous study has shown that the C allele of the VEGF rs2010963 polymorphism was associated with susceptibility to GD [46]. In the present study, the frequency of this allele also tended to be higher in GD patients relative to controls, but not significantly so (Table 5). We also found that the proportion of GD patients showing higher serum VEGF levels was increased in individuals with the rs3025039 CC genotype (Fig. 5D), was also shown in a previous study [49]. Furthermore, we found that the proportion of GD patients with larger goiters was increased in individuals with the same genotype (Fig. 2). Therefore, in subjects with the CC genotype, higher serum VEGF levels might increase angiogenesis in the thyroid glands of GD patients and result in enlargement of goiters.

In the case of VEGFR2, we also hypothesized that the frequency of the rs2071559 CC genotype would be higher in intractable GD patients than in GD patients in remission because individuals with this genotype showed lower serum VEGFR2 levels [50]. In accordance with this hypothesis, we found that the frequency of the CC genotype was higher in patients with intractable GD than control subjects (Table 8), and that the proportion of GD patients with larger goiters (>5.6 cm) was higher in individuals with the CC genotype than T carriers (Fig. 3). It is well known that VEGFR2 is expressed on endothelial cells [58] and can be detected in its soluble form in sera as soluble VEGFR2 (sVEGFR2). Although sVEGFR1 is known as an inhibitor of angiogenesis [59-61], recent studies have indicated that sVEGFR2 may trap VEGF in the peripheral blood and play a role as a natural cell regulator [62, 63]. Therefore, we suggested that individuals with the CC genotype of VEGFR2 rs2071559 may promote angiogenesis in thyroid glands through insufficient suppression of VEGF and induce enlargement of goiters to enhance the intractability of GD. However, there is the possibility that individuals with the CC genotype of this polymorphism also show lower full-length VEGFR2 expression. Further study is needed on this aspect.

In the VEGFR2 rs1870377 polymorphism, it has been reported that the T allele relates to higher binding affinity of VEGF to VEGFR2 [50]. As expected, we found

Fig. 5 Serum VEGF levels in each genotype of *VEGF* polymorphism.

We examined the difference in serum VEGF levels in each genotype of *VEGF* polymorphism by using healthy controls, untreated GD patients in remission, and untreated mild HD patients to avoid the effect of medical treatment. We analyzed the differences of serum VEGF levels (A and B) and the proportion of subjects with high serum VEGF levels (C and D). Analyzed by Student's *t* test except ^a analyzed by χ^2 test.

that the frequency of the TT genotype was higher in patients with intractable GD than those in remission (Table 8). Therefore, we suggested that individuals with the TT genotype of this polymorphism may exhibit enhanced angiogenesis associated with the intractability of GD.

Combined analysis showed that each genotype related to higher VEGF expression [44, 45, 47-49], risk of GD [46], lower serum VEGFR2 levels [50], and higher VEGF-VEGFR2 binding affinity [50] were more frequent in intractable GD patients than in GD patients in remission (Table 9). These findings suggest that the genetic combination of the interaction of VEGF-VEGFR2 ligation, especially combinations of gene polymorphisms with higher VEGF expression and with higher VEGF-VEGFR2 binding affinity, may be more important for the intractability of GD than each individ-

ual *VEGF* and *VEGFR2* polymorphism. Serum level of IL-6, which is a Th17-inducing cytokines, was significantly higher in patients with ulcerative colitis showing positive staining of VEGF [57]. We have previously reported that high proportion of Th17 cells may be associated with GD intractability. Therefore, VEGF-VEGFR2 ligation may be associated with GD intractability through Th17 differentiation.

On the other hand, serum VEGF levels were higher in AITD and HD patients and tended to be higher in GD patients than those in controls (Fig. 4). However, we also found that the frequencies of the I carrier and I allele of *VEGF* -2549 I/D polymorphism, which are associated with lower VEGF expression [44], were higher in severe HD patients relative to mild HD patients (Table 6). Since VEGF has a protective role against autoimmunity [56, 58], our findings suggest that HD patients who are genet-

ically disposed to lower rates of VEGF production might be susceptible to more severe HD than those with higher rates of production. Therefore, increase of serum VEGF in AITD patients might be a secondary phenomenon against autoimmune reaction. Furthermore, serum VEGF levels were higher in the CC genotype of *VEGF* rs2010963 than in G carriers (GG + GC genotypes) (Fig. 5C) and were higher in the CC genotype of *VEGF* rs3025039 than in T carriers (CT + TT genotypes) (Fig. 5D). Although they were not significant, serum VEGF levels were also higher in the DD genotype of the *VEGF* -2549 I/D polymorphism than in I carriers (DI + II genotypes) (Fig. 5A) and were higher in the GG genotype of the *VEGF* rs1570360 polymorphism than in the GA genotype (Fig. 5B). These results were generally consistent with previous reports that showed significant differences in VEGF measurements among each genotype of the four *VEGF* polymorphisms [44, 45, 47–49].

There are some limitations in study. We could not compare genotypes/alleles between groups with ophthalmopathy. This is because the prevalence of GD ophthalmopathy is too low in the Japanese population to analyze. We also could not compare genotypes/alleles relative to goiter size and family history because of sample numbers. In this study, goiter size was defined as the longitudinal length of the thyroid gland as measured using a vernier caliper. Therefore, we could not show calculated thyroid

volumes because of the difficulty of measuring this for all GD patients. Furthermore, despite the possibility that young controls could become patients in the future, we were unable to compare VEGF and VEGFR2 polymorphisms between controls and GD or HD patients using age-matched controls. However, there was no significant difference between controls and patients in this study.

In conclusion, *VEGF* polymorphisms with lower activity were associated with the severity of HD, and *VEGFR2* polymorphisms and the combination of *VEGF* and *VEGFR2* polymorphisms, with stronger interactions, were associated with the intractability of GD. *VEGF* and *VEGFR2* polymorphisms with higher activity were associated with goiter size at the onset of GD. *VEGF* and *VEGFR2* polymorphisms were associated with HD severity and GD intractability, respectively.

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Disclosure

The authors declare that they do not have any conflicts of interest.

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