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

Mami Okamoto¹, Mikio Watanabe¹, Naoya Inoue¹,², Kazane Ogawa¹, Yoh Hidaka³ and Yoshinori Iwatani¹

¹Department of Biomedical Informatics, Division of Health Sciences, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan
²Laboratory for Clinical Investigation, Osaka University Hospital, Osaka 565-0871, Japan

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

Submitted Oct. 20, 2019; Accepted Jan. 14, 2020 as EJ19-0480
Released online in J-STAGE as advance publication Feb. 20, 2020
Correspondence to: Yoshinori 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
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 μIU/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...
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 negative 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 ethylenediaminetetraacetic acid (EDTA)-treated whole blood cells with a commercially available kit (QIAGEN, Tokyo, Japan) according to manufacturer’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 x 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 x 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).

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

|                      | Controls | GD                  | HD                  |
|----------------------|----------|---------------------|---------------------|
|                      |          | Past clinical history of thyrotoxicosis | Diffuse goiter and positive TgAb or McAb |
|                      |          | with elevated TRAb | and/or McAb         |
|                      |          | Intractable        | Severe              |
|                      |          | 34.4 ± 14.0        | 38.5 ± 9.9          |
|                      |          | 33.6 ± 14.6        | 58.8 ± 12.2         |
|                      |          | 97 (83/14)         | 118 (97/21)         |
|                      |          | 58 (51/7)          | 81 (67/14)          |
| n (female/male)      | 117 (80/37) | 117 (80/37) | 117 (80/37) |
| Age of the time of sampling (years) | 33.7 ± 14.6 | 49.0 ± 15.0 | 49.1 ± 14.1 |
|                      | 33.6 ± 14.6 | 44.8 ± 15.3 | 62.7 ± 10.0 |
| Goiter size (cm)     | ND       | 4.9 ± 1.5          | 4.1 ± 1.0*          |
|                      |          | 4.2 ± 0.6          | 4.6 ± 1.3           |
| FT4 (ng/dL)          | 1.2 ± 0.3 | 1.2 ± 0.3          | 1.3 ± 0.3**         |
|                      |          | 1.2 ± 0.2          | 1.2 ± 0.2           |
| TSH (mIU/mL)         | 1.6 ± 1.0 | 1.7 ± 1.4          | 4.1 ± 10.3          |
|                      |          | 1.8 ± 1.2          | 2.8 ± 1.9           |
| TRAb (IU/L)          | <2.0     | 13.6 ± 60.1<2.0    | 6.6 ± 3.2****       |
|                      |          | 13.6 ± 60.1<2.0    | 1.3 ± 2.4           |
| TgAb (2^a × 100)a    | Negative | 2.8 ± 3.0          | 5.4 ± 3.0           |
|                      |          | 2.9 ± 3.2          | 3.5 ± 2.8           |
| McAb (2^a × 100)a    | Negative | 4.4 ± 2.7          | 5.5 ± 2.0           |
|                      |          | 5.5 ± 2.0          |                     |
| Current treatment    | None     | MMI or PTU         | L-thyroxine         |
| Current dose of anti-thyroid drug (mg/day)b (range) | None | 14.5 ± 20.8 (2.5–150) | None | None |
|                      |          | 14.5 ± 20.8 (2.5–150) | 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
* When the titer of TgAb or McAb was 25,600, it was expressed as 2^a × 100, b Doses were expressed as the comparable dose of MMI (50 mg of PTU was conveted 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)
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.

| Gene  | Polymorphism | Region       | Effect to expression                                      |
|-------|--------------|--------------|-----------------------------------------------------------|
| VEGF  | –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)                    |
| VEGFR2| 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) VEGF rs2071559, and (F) VEGFR rs1870377 polymorphisms. Detailed information on each genotype is shown in Table 3.
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 mild HD ($p = 0.0012$).

Statistical analysis
We used the $\chi^2$ 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 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 |
|------|--------------|------------|----------------|------------------------------|-------------------------------------|
| VEGF | rs1570360    | 5'-TCCTGCTCCCTCCTCGCAATG-3' <br> 5'-GGGGGAGCACGGCTCATC-3' | 95°C for 5 min <br> (95°C for 30 sec, 61.1°C for 30 sec, 72°C for 30 sec) × 35 cycle <br> 72°C for 5 min | PCR-RFLP (MnlI) | AA genotype: 206 bp <br> AG genotype: 206 bp + 187 bp + 19 bp <br> GG genotype: 187 bp + 19 bp |
|      | rs2010963    | 5'-GGAGATTTGCTCTACTTCCTCCAAGGCTGCAATG-3' | 95°C for 5 min <br> (95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec) × 30 cycle <br> 72°C for 5 min | PCR-RFLP (BsmFI) | CC genotype: 256 bp <br> GC genotype: 256 bp + 145 bp + 111 bp <br> GG genotype: 145 bp + 111 bp |
|      | rs3025039    | 5'-GATACAGAAACACGCTGCC-3' <br> 5'-CGGGCTCGTGATATAGCAGAG-3' | 95°C for 5 min <br> (95°C for 30 sec, 60.6°C for 30 sec, 72°C for 30 sec) × 35 cycle <br> 72°C for 5 min | PCR-RFLP (NlaIII) | CC genotype: 219 bp <br> CT genotype: 219 bp + 160 bp + 59 bp <br> TT genotype: 160 bp + 59 bp |
| VEGFR2 | rs2071559   | 5'-CAAACCTTTCTAGGGCTTCTTGCTGTG-3' <br> 5'-AGCCCAAGGGAGAACCGGATA-3' | 95°C for 5 min <br> (95°C for 30 sec, 61°C for 30 sec, 72°C for 30 sec) × 30 cycle <br> 72°C for 5 min | PCR-RFLP (BsmI) | TT genotype: 290 bp <br> CT genotype: 290 bp + 174 bp + 116 bp <br> CC genotype: 174 bp + 116 bp |
|      | rs1870377    | 5'-GCCTCACATATTATGTACCATCCTC3' <br> 5'-CCTCCTGTATCCTGAAATGATC-3' | 95°C for 5 min <br> (95°C for 30 sec, 60.5°C for 30 sec, 72°C for 30 sec) × 29 cycle <br> 72°C for 5 min | PCR-RFLP (AluI) | AA genotype: 404 bp <br> AT genotype: 404 bp + 213 bp + 191 bp <br> TT genotype: 191 bp + 213 bp
levels of TRAb among each genotype. Student’s t test, the Mann-Whitney U test, and the χ² 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-

---

**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 |
| n (female/male)      | 14 (11/3) | 16 (14/2) | 16 (14/2) |
| The age of onset (years) | 34.4 ± 15.2 | 35.4 ± 13.7 | 38.3 ± 9.9 |
| Age at the time of sampling (years) | 51.1 ± 16.7 | 54.1 ± 11.9 | 54.5 ± 10.7 |
| Goiter size (cm)     | ND       | 5.0 ± 1.0* | 3.8 ± 0.4 |
| Free T4 (ng/dL)      | 1.2 ± 0.3 | 1.1 ± 0.1 | 1.2 ± 0.2 |
| TSH (μU/mL)          | 1.6 ± 1.0 | 1.9 ± 1.1 | 1.6 ± 0.6 |
| TRAb (IU/L)          | <2.0     | 13.9 ± 22.4 | <2.0 |
| Current treatment    | None     | MMI or PTU | None |
| Current dose of antithyroid drug (mg/day) | None | 14.1 ± 11.8 (1.25–30) | None |
| Current dose of L-thyroxine (μg/day) | None | None | 84.3 ± 31.1 (37.5–150) |

Data are expressed as mean ± standard deviation
ND, not determined; PTU, propylthiouracil; MMI, methimazole
* 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)
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 ($p = 0.0427, 0.0222, 0.0491, 0.0326, \text{ and } 0.0342$, respectively) (Table 9).

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

| VEGF –2549 I/D     | Control | All patients with AITD | All patients with GD | All patients with HD |
|--------------------|---------|------------------------|----------------------|---------------------|
| II                 | 11 (11.22%) | 33 (7.91%)             | 15 (7.94%)           | 18 (7.9%)           |
| ID                 | 35 (35.72%) | 157 (37.65%)           | 73 (38.62%)          | 84 (36.84%)         |
| 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%)           | 101 (53.44%)         | 126 (55.26%)        |
| II                 | 11 (11.22%) | 33 (7.91%)             | 15 (7.94%)           | 18 (7.9%)           |
| ID + DD            | 87 (88.78%) | 384 (92.09%)           | 174 (92.06%)         | 210 (92.1%)         |
| I allele           | 57 (29.08%) | 223 (26.74%)           | 103 (27.25%)         | 120 (26.32%)        |
| D allele           | 139 (70.92%) | 611 (73.26%)           | 275 (72.75%)         | 336 (73.68%)        |

| VEGF rs1570360    | AA      | 3 (3%)                  | 2 (0.49%)            | 1 (0.54%)           | 1 (0.45%)           |
|                   | AG      | 24 (24%)                | 88 (21.57%)          | 44 (23.91%)         | 46 (20.54%)         |
|                   | GG      | 73 (73%)                | 318 (77.94%)         | 183 (99.46%)        | 178 (79.46%)        |
| AA + AG           | 27 (27%) | 90 (22.06%)             | 44 (23.91%)          | 46 (20.54%)         | 178 (79.46%)        |
| GG                 | 73 (73%) | 318 (77.94%)            | 140 (76.09%)         | 178 (79.46%)        |                     |
| AA                 | 3 (3%)  | 2 (0.49%)               | 1 (0.54%)            | 1 (0.45%)           |                     |
| AG + GG           | 97 (97%) | 406 (99.51%)            | 183 (99.46%)         | 223 (99.55%)        |                     |
| A allele           | 30 (15%) | 92 (11.27%)             | 45 (12.23%)          | 47 (10.49%)         |                     |
| G allele           | 170 (85%) | 724 (88.73%)            | 323 (87.77%)         | 401 (89.51%)        |                     |

| VEGF rs2010963    | CC      | 18 (16.36%)             | 95 (22.78%)          | 43 (23.12%)         | 52 (22.51%)         |
|                   | GC      | 56 (50.91%)             | 202 (48.44%)         | 95 (51.07%)         | 107 (46.32%)        |
|                   | 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%)            | 48 (25.81%)          | 72 (31.17%)         |                     |
| CC                 | 18 (16.36%) | 95 (22.78%)             | 43 (23.12%)          | 52 (22.51%)         |                     |
| GC + GG           | 92 (83.64%) | 322 (77.22%)            | 143 (76.88%)         | 179 (77.49%)        |                     |
| C allele           | 92 (41.82%) | 392 (83.64%)            | 181 (48.66%)         | 211 (45.67%)        |                     |
| G allele           | 128 (58.18%) | 442 (55.36%)            | 191 (51.34%)         | 251 (54.33%)        |                     |

| VEGF rs3025039    | CC      | 67 (65.69%)             | 275 (66.1%)          | 121 (64.02%)        | 154 (67.84%)        |
|                   | CT      | 29 (28.43%)             | 123 (29.57%)         | 58 (30.69%)         | 65 (28.64%)         |
|                   | 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%)              | 10 (5.29%)           | 8 (3.52%)           |                     |
| CC                 | 67 (65.69%) | 275 (66.1%)             | 121 (64.02%)         | 154 (67.84%)        |                     |
| CT + TT           | 35 (34.31%) | 141 (33.9%)             | 68 (35.98%)          | 73 (32.16%)         |                     |
| C allele           | 163 (79.9%) | 673 (80.89%)            | 300 (79.37%)         | 373 (82.16%)        |                     |
| T allele           | 41 (20.1%)  | 159 (19.11%)            | 78 (20.63%)          | 81 (17.84%)         |                     |

Analyzed by $\chi^2$ test except * analyzed by Fisher’s exact test. a vs. control, NS: not significant.
Table 6  Genotype and allele frequencies of VEGF polymorphisms genotyped in this study in patients with HD and GD

| VEGF rs1570360 | Control | GD | HD |
|----------------|---------|----|----|
|               | Intractable | In remission | Severe | Mild |
| II            | 11 (11.22%) | 9 (9.89%) | 3 (3.26%) | 11 (9.82%) | 4 (5.26%) |
| ID            | 35 (35.72%) | 38 (41.76%) | 21 (36.84%) | 44 (39.29%) | 22 (28.95%) | NS\(^a\) |
| 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%) | 55 (49.11%) | 26 (34.21%) | 0.043\(^c\) |
| ID + DD       | 87 (88.78%) | 82 (90.11%) | 54 (94.74%) | 101 (90.18%) | 72 (94.74%) | NS\(^c\) |
| II            | 11 (11.22%) | 9 (9.89%) | 3 (5.26%) | 11 (9.82%) | 4 (5.26%) |
| ID + DD       | 87 (88.78%) | 82 (90.11%) | 54 (94.74%) | 101 (90.18%) | 72 (94.74%) | NS\(^c\) |
| I allele      | 57 (29.08%) | 56 (30.77%) | 27 (23.68%) | 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%) |
| VEGF rs2010963 |         |       |     |       |       |
|               | Intractable | In remission | Severe | Mild |
| AA            | 3 (3%) | 1 (1.12%) | 0 (0%) | 1 (0.9%) | 0 (0%) |
| AG            | 24 (24%) | 23 (25.84%) | 11 (20%) | 26 (23.42%) | 11 (15.07%) | NS\(^a\) |
| GG            | 73 (73%) | 65 (73.04%) | 44 (80%) | 84 (75.68%) | 62 (84.93%) |
| AA + AG       | 27 (27%) | 24 (26.96%) | 11 (20%) | 27 (24.32%) | 11 (15.07%) | NS\(^a\) |
| GG            | 73 (73%) | 65 (73.04%) | 44 (80%) | 84 (75.68%) | 62 (84.93%) |
| AA            | 3 (3%) | 1 (1.12%) | 0 (0%) | 1 (0.9%) | 0 (0%) |
| AG + GG       | 97 (97%) | 88 (98.88%) | 55 (100%) | 110 (99.1%) | 73 (100%) | NS\(^a\) |
| A allele      | 30 (15%) | 25 (14.04%) | 11 (10%) | 28 (12.61%) | 11 (7.53%) | NS\(^a\) |
| G allele      | 170 (85%) | 153 (85.96%) | 99 (90%) | 194 (87.39%) | 135 (92.47%) |
| VEGF rs3025039 |         |       |     |       |       |
|               | Intractable | In remission | Severe | Mild |
| CC            | 18 (16.36%) | 19 (21.11%) | 12 (21.43%) | 22 (19.3%) | 24 (31.17%) |
| GC            | 56 (50.91%) | 43 (47.78%) | 31 (55.36%) | 54 (47.37%) | 29 (37.66%) | NS\(^a\) |
| 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%) | 76 (66.67%) | 53 (68.83%) | NS\(^a\) |
| 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%) | 22 (19.3%) | 24 (31.17%) |
| 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%) | 98 (42.98%) | 77 (50%) | NS\(^a\) |
| G allele      | 128 (58.18%) | 99 (55%) | 57 (50.89%) | 130 (57.02%) | 77 (50%) |

**Note:** Analyzed by \( \chi^2 \) 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.
Serum VEGF levels

Serum VEGF levels were higher in HD and AITD patients than those in control subjects \( (p = 0.0154, 0.0208, \text{respectively}) \) (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 7  Genotype and allele frequencies of the VEGFR2 polymorphisms in patients with AITD and in control subjects

| Genotype       | Control | All patients with AITD | All patients with GD | All patients with HD |
|----------------|---------|------------------------|----------------------|----------------------|
| VEGFR2 rs2071559 |         |                        |                      |                      |
| CC             | 3 (2.89%) | 28 (6.68%)             | 13 (6.99%)           | 15 (6.44%)           |
| CT             | 35 (33.65%) | 149 (35.56%)       | NS\(^a\)            | NS\(^a\)             |
| TT             | 66 (63.46%) | 242 (57.76%)       | 108 (58.06%)        | 134 (57.51%)         |
| CC + CT        | 38 (36.54%) | 177 (42.24%)       | NS\(^a\)            | NS\(^a\)             |
| TT             | 66 (63.46%) | 242 (57.76%)       | 108 (58.06%)        | 134 (57.51%)         |
| C allele       | 41 (19.71%) | 205 (24.46%)       | NS\(^a\)            | NS\(^a\)             |
| T allele       | 167 (80.29%) | 633 (75.54%)       | 281 (75.54%)        | 352 (75.54%)         |
| VEGFR2 rs1870377 |         |                        |                      |                      |
| AA             | 12 (11.88%) | 52 (12.59%)        | 24 (12.7%)           | 28 (12.5%)           |
| AT             | 54 (53.47%) | 220 (53.27%)      | NS\(^a\)            | NS\(^a\)             |
| TT             | 35 (34.65%) | 141 (34.14%)      | 58 (30.69%)          | 83 (37.05%)          |
| AA + AT        | 66 (65.35%) | 272 (65.86%)      | NS\(^a\)            | NS\(^a\)             |
| TT             | 35 (34.65%) | 141 (34.14%)      | 58 (30.69%)          | 83 (37.05%)          |
| AA             | 12 (11.88%) | 52 (12.59%)        | 24 (12.7%)           | 28 (12.5%)           |
| AT + TT        | 89 (88.12%) | 361 (87.41%)      | NS\(^a\)            | NS\(^a\)             |
| A allele       | 78 (38.61%) | 324 (39.23%)      | 155 (41.01%)         | 169 (37.72%)         |
| T allele       | 124 (61.39%) | 502 (60.77%)      | 223 (58.99%)         | 279 (62.28%)         |

Analyzed by \(\chi^2\) 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 \(\chi^2\) 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 \(\chi^2\) 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

### 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%) | NSb  |
|                   | CT      | 35 (33.65%)         | 29 (32.95%) | 18 (31.58%) | 41 (35.96%) | 24 (32.43%) | NSc  |
|                   | 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%) | NSb  |
|                   | TT      | 66 (63.46%)         | 50 (56.82%) | 38 (66.67%) | 65 (57.02%) | 44 (59.46%) |                                  |
| VEGFR2 rs1870377  | AA      | 12 (11.88%)         | 10 (10.75%) | 8 (14.03%) | 11 (9.73%) | 10 (14.08%) |                                  |
|                   | 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 (35.45%) | 28 (39.44%) |                                  |
| AA + AT           | 66 (63.55%) | 57 (61.29%) | 44 (77.19%) | 73 (64.6%) | 43 (60.56%) | NSc  |
|                   | TT      | 35 (34.65%)         | 36 (38.71%) | 13 (22.81%) | 40 (35.45%) | 28 (39.44%) |                                  |
| VEGFR2 rs1570360  | CC      | 12 (11.88%)         | 10 (10.75%) | 8 (14.03%) | 11 (9.73%) | 10 (14.08%) |                                  |
|                   | CT      | 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 (35.45%) | 28 (39.44%) |                                  |
| CC + CT           | 38 (36.54%) | 38 (43.18%) | 19 (33.33%) | NSb  |
|                   | TT      | 66 (63.46%)         | 50 (56.82%) | 38 (66.67%) | 65 (57.02%) | 44 (59.46%) |                                  |
| C allele          | 41 (19.71%) | 47 (26.7%)  | 20 (17.54%) | 57 (25%)  | 36 (24.32%) |                                  |
| T allele          | 167 (80.29%) | 129 (73.3%) | 94 (82.46%) | 171 (75%) | 112 (75.68%) |                                  |

**Analyzed by χ² 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 | Control | GD                  | HD       |
|--------------------------------|---------|---------------------|----------|
|                                |         | Intractable         | In remission | Severe | Mild |
| VEGF rs1570360/VEGFR2 rs2071559| GG/CC   | 7 (8.14%)           | 0 (0%)    | 55 (100%) | 0.0427* |
|                                | others  | 79 (91.86%)         |          |                                  |
| VEGF rs2010963/VEGFR2 rs2071559| CC + CG | 8 (9.3%)            | 0 (0%)    | 56 (100%) | 0.0222* |
|                                | CC      | 78 (90.7%)          |          |                                  |
| VEGF rs3025039/VEGFR2 rs2071559| CC + CT | 9 (10.71%)          | 1 (1.75%) | 56 (98.25%) | 0.0491* |
|                                | CC      | 75 (89.29%)         |          |                                  |
| VEGF –2549 I/D/VEGFR2 rs1870377| DD + ID | 33 (37.93%)         | 12 (21.05%) | 45 (78.95%) | 0.0326 |
|                                | TT      | 54 (62.07%)         |          |                                  |
| VEGF rs1570360/VEGFR2 rs1870377| GG + GA | 36 (40.91%)         | 13 (23.64%) | 42 (76.36%) | 0.0342 |
|                                | others  | 52 (59.09%)         |          |                                  |

**Analyzed by χ² test except a analyzed by Fisher’s exact 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 rs3025039 than that in T carriers (CT + TT 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 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
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 individual 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 inAITD 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-

![Graphs showing serum VEGF levels in each genotype of VEGF polymorphism.](image)
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 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.

Acknowledgements

This work was supported by JSPS KAKENHI grant nos. JP17H04111, JP17K15774, and JP19H04048.

Disclosure

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

References

1. Weetman AP (2013) The immunopathogenesis of chronic autoimmune thyroiditis one century after hashimoto. *Eur Thyroid J* 1: 243–250.
2. Menconi F, Marcocci C, Marino M (2014) Diagnosis and classification of Graves’ disease. *Autoimmun Rev* 13: 398–402.
3. McLeod DS, Cooper DS (2012) The incidence and prevalence of thyroid autoimmunity. *Endocrine* 42: 252–265.
4. Brix TH, Hegedus L (2012) Twin studies as a model for exploring the aetiology of autoimmune thyroid disease. *Clin Endocrinol (Oxf)* 76: 457–464.
5. Coppede F (2017) Epigenetics and autoimmune thyroid diseases. *Front Endocrinol (Lausanne)* 8: 149.
6. Eschler DC, Hasham A, Tomer Y (2011) Cutting edge: the etiology of autoimmune thyroid diseases. *Clin Rev Allergy Immunol* 41: 190–197.
7. Ito C, Watanabe M, Okuda N, Watanabe C, Iwatani Y (2006) Association between the severity of Hashimoto’s disease and the functional +874A/T polymorphism in the interferon-gamma gene. *Endocr J* 53: 473–478.
8. Nanba T, Watanabe M, Akamizu T, Iwatani Y (2008) The –590CC genotype in the IL4 gene as a strong predictive factor for the development of hypothyroidism in Hashimoto disease. *Clin Chem* 54: 621–623.
9. Nanba T, Watanabe M, Inoue N, Iwatani Y (2009) Increases of the Th1/Th2 cell ratio in severe Hashimoto’s disease and in the proportion of Th17 cells in intractable Graves’ disease. *Thyroid* 19: 495–501.
10. Yamada H, Watanabe M, Nanba T, Akamizu T, Iwatani Y (2008) The +869T/C polymorphism in the transforming growth factor-beta1 gene is associated with the severity and intractability of autoimmune thyroid disease. *Clin Exp Immunol* 151: 379–382.
11. Hayashi F, Watanabe M, Nanba T, Inoue N, Akamizu T, et al. (2009) Association of the –31C/T functional polymorphism in the interleukin-1beta gene with the intractability of Graves’ disease and the proportion of T helper type 17 cells. *Clin Exp Immunol* 158: 281–286.
12. Castagnone D, Rivolta R, Rescalli S, Baldini MI, Tozzi R, et al. (1996) Color Doppler sonography in Graves’ disease: value in assessing activity of disease and predicting outcome. *AJR Am J Roentgenol* 166: 203–207.
13. Ralls PW, Mayekawa DS, Lee KP, Colletti PM, Radin DR, et al. (1988) Color-flow Doppler sonography in Graves disease: “thyroid inferno”. *AJR Am J Roentgenol* 150: 781–784.
14. Nagura S, Katoh R, Miyagi E, Shibuya M, Kawaai A (2001) Expression of vascular endothelial growth factor (VEGF) and VEGF receptor-1 (Flt-1) in Graves disease possibly correlated with increased vascular density. *Hum Pathol* 32: 10–17.

15. Iitaka M, Miura S, Yamanaka K, Kawasaki S, Kitahama S, et al. (1998) Increased serum vascular endothelial growth factor levels and intrathyroidal vascular area in patients with Graves’ disease and Hashimoto’s thyroiditis. *J Clin Endocrinol Metab* 83: 3908–3912.

16. Laurberg P, Buchholtz Hansen PE, Iversen E, Eskjaer Jensen S, Weeke J (1986) Goitre size and outcome of medical treatment of Graves’ disease. *Acta Endocrinol (Copenh)* 111: 39–43.

17. Vitti P, Ragot T, Chiovato L, Pallini S, Santini F, et al. (1997) Clinical features of patients with Graves’ disease undergoing remission after antithyroid drug treatment. *Thyroid* 7: 369–375.

18. Winsa B, Dahlberg A, Jansson R, Agren H, Karlsson FA (1990) Factors influencing the outcome of thyrostatic drug therapy in Graves’ disease. *Acta Endocrinol (Copenhagen)* 122: 722–728.

19. Schleusener H, Schwander J, Fischer C, Holle R, Holl G, et al. (1989) Prospective multicentre study on the prediction of relapse after antithyroid drug treatment in patients with Graves’ disease. *Acta Endocrinol (Copenhagen)* 120: 689–701.

20. Kim TY, Park YJ, Park DJ, Chung HK, Kim WB, et al. (2005) Role of the vascular endothelial growth factor/vascular permeability factor in serum of children with thyrotoxicosis. *J Clin Endocrinol Metab* 88: 117–124.

21. Allahabadia A, Daykin J, Holder RL, Sheppard MC, Gough SC, et al. (2000) Age and gender predict the outcome of treatment for Graves’ hyperthyroidism. *J Clin Endocrinol Metab* 85: 1038–1042.

22. Glaser NS, Styne DM (1997) Predictors of early remission of hyperthyroidism in children. *J Clin Endocrinol Metab* 82: 1719–1726.

23. Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 23: 1011–1027.

24. Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 25: 581–611.

25. Namiki A, Brogi E, Kearney M, Kim EA, Wu T, et al. (1995) Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *J Biol Chem* 270: 31189–31195.

26. Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR (1992) Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell* 3: 211–220.

27. Kondo S, Asano M, Matsuo K, Ohmori I, Suzuki H (1994) Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients. *Biochim Biophys Acta* 1221: 211–214.

28. Poon RT, Fan ST, Wong J (2001) Clinical implications of circulating angiogenic factors in cancer patients. *J Clin Oncol* 19: 1207–1225.

29. Carvalho JF, Blank M, Shoenfeld Y (2007) Vascular endothelial growth factor (VEGF) in autoimmune diseases. *J Clin Immunol* 27: 246–256.

30. Ozgonenel L, Cetin E, Tutun S, Tonbaklar P, Aral H, et al. (2010) The relation of serum vascular endothelial growth factor level with disease duration and activity in patients with rheumatoid arthritis. *Clin Rheumatol* 29: 473–477.

31. Mekl AR, Al-Shobaili H (2014) Serum vascular endothelial growth factor, transforming growth factor beta1, and nitric oxide levels in patients with psoriasis vulgaris: their correlation to disease severity. *J Clin Lab Anal* 28: 496–501.

32. Kuryliszyn-Moskal A, Klimentiu PA, Sierakowski S, Ciolkiewicz M (2007) Vascular endothelial growth factor in systemic lupus erythematosus: relationship to disease activity, systemic organ manifestation, and nailfold capillaroscopic abnormalities. *Arch Immunol Ther Exp (Warsz)* 55: 179–185.

33. Vural P, Degirmencioglu S, Erden S, Gelicnik C (2009) The relationship between transforming growth factor-beta1, vascular endothelial growth factor, nitric oxide and Hashimoto’s thyroiditis. *Int Immunopharmacol* 9: 212–215.

34. Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. *Nat Med* 9: 669–676.

35. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, et al. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62–66.

36. Bernatchez PN, Soker S, Sirois MG (1999) Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent. *J Biol Chem* 274: 31047–31054.

37. McMahon G (2000) VEGF receptor signaling in tumor angiogenesis. *Oncologist* 5 Suppl 1: 3–10.

38. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A (1994) Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature* 367: 576–579.

39. Herold-Mende C, Steiner HH, Andl T, Riede D, Buttler A, et al. (1999) Expression and functional significance of vascular endothelial growth factor receptors in human tumor cells. *Lab Invest* 79: 1573–1582.

40. Guo S, Colbert LS, Fuller M, Zhang Y, Gonzalez-Perez RR (2010) Vascular endothelial growth factor receptor-2 in breast cancer. *Biochim Biophys Acta* 1806: 108–121.

41. Chorley BN, Wang X, Campbell MR, Pittman GS, Noureldine MA, et al. (2008) Discovery and verification of functional single nucleotide polymorphisms in regulatory genomic regions: current and developing technologies. *Mutat Res* 659: 147–157.

42. Albert PR (2011) What is a functional genetic polymorphism? Defining classes of functionality. *J Psychiatry Neurosci* 36: 363–365.

43. Breunis WB, Bizeveld MH, Geissler J, Ottenkamp J,
Kuipers IM, et al. (2006) Vascular endothelial growth factor gene haplotypes in Kawasaki disease. *Arthritis Rheum* 54: 1588–1594.

44. Yang B, Cross DF, Ollerenshaw M, Millward BA, Demaine AG (2003) Polymorphisms of the vascular endothelial growth factor gene and susceptibility to diabetic microvascular complications in patients with type 1 diabetes mellitus. *J Am Soc Nephrol* 13: 260–264.

45. Shahbazi M, Fryer AA, Pravica V, Brogan IJ, Ramsay HM, et al. (2002) Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection. *J Am Soc Nephrol* 13: 260–264.

46. Vural P, Baki M, Dogru-Abbasoglu S, Ozderya A, Karadag B, et al. (2012) Vascular endothelial growth factor gene polymorphisms increase the risk of developing Graves’ disease. *Int Immunopharmacol* 14: 133–137.

47. Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, et al. (2002) A common polymorphism in the 5′-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes. *Diabetes* 51: 1635–1639.

48. Vailati FB, Crispim D, Sortica DA, Souza BM, Brondani LA, et al. (2012) The C allele of –634G/C polymorphism in the VEGFA gene is associated with increased VEGFA gene expression in human retinal tissue. *Invest Ophthalmol Vis Sci* 53: 6411–6415.

49. Krippl P, Langsenlehner U, Renner W, Yazdani-Biuki B, Wolf G, et al. (2003) A common 936 C/T gene polymorphism of vascular endothelial growth factor is associated with decreased breast cancer risk. *Int J Cancer* 106: 468–471.

50. Wang Y, Zheng Y, Zhang W, Yu H, Lou K, et al. (2007) Polymorphisms of KDR gene are associated with coronary heart disease. *J Am Coll Cardiol* 50: 760–767.

51. Gao Y, Ma P, He Y, Liu Y, Jiang Y (2016) Genetic Variations of Kinase Inserts Domain Receptor (KDR) gene are associated with the risk of astrocytomas. *Mol Neurobiol* 53: 2541–2549.

52. Watanabe M, Yamamoto N, Maruoka H, Tamai H, Matsuzaka F, et al. (2002) Independent involvement of CD8+ CD25+ cells and thyroid autoantibodies in disease severity of Hashimoto’s disease. *Thyroid* 12: 801–808.

53. Brogan IJ, Khan N, Isaac K, Hutchinson JA, Pravica V, et al. (1999) Novel polymorphisms in the promoter and 5′ UTR regions of the human vascular endothelial growth factor gene. *Hum Immunol* 60: 1245–1249.

54. Del Bo R, Scarlato M, Ghezzi S, Maestroni A, Sjolind L, et al. (2006) VEGF gene variability and type 1 diabetes: evidence for a protective role. *Immunogenetics* 58: 107–112.

55. Bagchi M, Kim LA, Boucher J, Walshe TE, Kahn CR, et al. (2013) Vascular endothelial growth factor is important for brown adipose tissue development and maintenance. *FASEB J* 27: 3257–3271.

56. Lin W (2017) Neuroprotective effects of vascular endothelial growth factor A in the experimental autoimmune encephalomyelitis model of multiple sclerosis. *Neural Regen Res* 12: 70–71.

57. Zdravkovic ND, Jovanovic IP, Radosavljevic GD, Arsenijevic AN, Zdravkovic ND, et al. (2014) Potential dual immunomodulatory role of VEGF in ulcerative colitis and colorectal carcinoma. *Int J Med Sci* 11: 936–947.

58. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT (1993) Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci U S A* 90: 7533–7537.

59. Ye C, Feng C, Wang S, Wang KZ, Huang N, et al. (2004) sFlt-1 gene therapy of follicular thyroid carcinoma. *Endocrinology* 145: 817–822.

60. Kendall RL, Thomas KA (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A* 90: 10705–10709.

61. Toi M, Bando H, Ogawa T, Muta M, Hornig C, et al. (2002) Significance of vascular endothelial growth factor (VEGF)/soluble VEGF receptor-1 relationship in breast cancer. *Int J Cancer* 98: 14–18.

62. Kou B, Li Y, Zhang L, Zhu G, Wang X, et al. (2004) In vivo inhibition of tumor angiogenesis by a soluble VEGFR-2 fragment. *Exp Mol Pathol* 76: 129–137.

63. Ebos JM, Lee CR, Bogdanovic E, Alami J, Van Slyke P, et al. (2008) Vascular endothelial growth factor-mediated decrease in plasma soluble vascular endothelial growth factor receptor-2 levels as a surrogate biomarker for tumor growth. *Cancer Res* 68: 521–529.