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Serum semaphorin 5A in Hashimoto's thyroiditis

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Elevated semaphorin 5A in patients with Hashimoto's thyroiditis: a case–control study

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Abstract

Objective: Hashimoto's thyroiditis (HT) is characterized by elevated specific auto-antibodies, including TgAb and TPOAb. Increasing evidence has demonstrated the essential role of Th17 cells in HT. However, the underlying mechanism is still unclear. Semaphorin 5A (Sema 5A) is involved in several autoimmune diseases through the regulation of immune cells. The aim of the present study was to explore the role of Sema 5A in HT.

Methods: We measured serum Sema 5A levels in HT ($n = 92$) and healthy controls ($n = 111$) by enzyme-linked immunosorbent assay (ELISA). RNA levels of Sema 5A and their receptors (plexin-A1 and plexin-B3), as well as several cytokines (IFN-γ, IL-4 and IL-17), were detected by real-time polymerase chain reaction in peripheral blood mononuclear cells from 23 patients with HT and 31 controls. In addition, we investigated the relationship between serum Sema 5A and HT.

Results: Serum Sema 5A in HT increased significantly compared with healthy controls ($P < 0.001$). Moreover, serum Sema 5A levels were positively correlated with TgAb ($r = 0.511$, $P < 0.001$), TPOAb ($r = 0.423$, $P < 0.001$), TSH ($r = 0.349$, $P < 0.001$) and IL-17 mRNA expression ($r = 0.442$, $P < 0.001$). Increased Sema 5A RNA expression was observed ($P = 0.041$) in HT compared with controls. In receiver-operating characteristic (ROC) analysis, serum Sema 5A predicted HT with a sensitivity of 79.35% and specificity of 96.40%, and the area under the curve of the ROC curve was 0.836 (95% CI: 0.778–0.884, $P < 0.001$).

Conclusions: These data demonstrated elevated serum Sema 5A in HT patients for the first time. Serum Sema 5A levels were correlated with thyroid auto-antibodies and IL-17 mRNA expression. Sema 5A may be involved in immune response of HT patients.

Introduction

Hashimoto's thyroiditis (HT), the most common type of thyroiditis and the most frequent cause of hypothyroidism (1), is characterized by high levels of thyroid auto-antibodies (antithyrogloblin antibody, TgAb and thyroperoxidase antibody, TPOAb) and diffuse goiter. Massive lymphocytic infiltration in the thyroid gland and varying degrees of fibrosis or atrophy leading to the destruction of thyroid follicle were confirmed by pathological analysis (2). Meanwhile, HT has also been found to coexist with other autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythaematosus (SLE) (3, 4, 5).

Previous studies have demonstrated that HT development involves a complex interplay between

genetic factors and environmental components (6). However, the underlying pathogenesis of HT is still unclear (7). Recent studies have revealed that elevated serum IL-17 and Th17 levels in HT patients were correlated with pathogenesis (8, 9, 10). Moreover, Th17 cells were shown to be responsible for other autoimmune diseases, such as SLE and RA, which are traditionally believed to be mediated by Th1 cells (11, 12). Consistent with these findings, Th17 cells were also found to be involved in the development of iodine-induced autoimmune thyroiditis in NOD-H2^{h4} mice (13).

The semaphorin family is composed of eight classes and characterized by a conserved '*Sema*' domain at the N-terminus (14, 15). As a family of transmembrane proteins and secreted proteins, semaphorins were initially identified as the axonal signaling molecules guiding neuronal axons to appropriate target cells in the nervous system (16). Recent studies revealed that different classes of the semaphorin family may play different roles by binding with their receptors (15). Plexins and neuropilins were identified as the primary receptors of semaphorins (17, 18). Several semaphorins and their cognate receptors are also involved in non-neuronal physiological processes, such as vasculogenesis (19), tumourigenesis (20) and immune cell regulation (21). However, the role of sub-family V in the immune response is still unclear.

Semaphorin 5A (Sema 5A), one of the sub-family V of semaphorins, contains a specific seven-repeat domain of thrombospondin in its extracellular domain (22). A secreted form of Sema 5A has been previously identified (23). Receptors of Sema 5A include plexin-A1 and plexin-B3 (24). Recent studies have revealed the major role of Sema 5A in innate immune responses via induction of TNF-α and IL-8 (25). Subsequently, the role of Sema 5A in autoimmune diseases has received increasing attention. One recent study found elevated secreted Sema 5A levels in patients with RA compared with healthy controls (26). In addition, strongly increased T cells and natural killer (NK) cells, as well as the pro-inflammatory cytokine secretion of T cells and NK cells, were observed after stimulation with soluble Sema 5A (26). Another study identified an elevated Sema 5A expression of mRNA in peripheral blood mononuclear cells (PBMCs) of patients with chronic primary immune thrombocytopaenia (ITP) (27). Moreover, a recent study conducted by Du *et al.* demonstrated elevated serum Sema 5A in SLE (28). However, the roles of Sema 5A in HT have not been determined.

In the present study, the serum Sema 5A level and mRNA expression of Sema 5A and its receptors in PBMCs of HT patients and healthy controls were detected. In addition, we further investigated the relationship between serum Sema 5A and HT.

Materials and methods

Patients and samples

A total of 92 newly diagnosed HT patients, including 29 patients with subclinical hypothyroidism (sHT) and 63 patients with euthyroidism (eHT), from the outpatient clinics of the Endocrine Department, the First Hospital of China Medical University, were enrolled in the present study. The diagnosis of HT was based on diffuse goiter and elevated thyroid auto-antibodies. In addition, 111 volunteers, who were matched for age, sex and body mass index (BMI), were recruited as healthy controls. All subjects with other autoimmune diseases (such as SLE, RA, multiple sclerosis and type I diabetes), acute and/or chronic infection, serious heart disease and pregnancy were excluded. None of them had taken levothyroxine. All participants provided written informed consent prior to enrolment. The study was approved by the Ethics Institutional Review Board of China Medical University prior to initiating subject recruitment.

After 8 h of fasting, 5 mL elbow venous blood samples were obtained. Serum was obtained after centrifugation and then stored at −80°C until use. The PBMCs from 23 with eHT of 92 patients and 31 of 111 healthy controls were separated from EDTA anti-coagulated whole blood.

Measurement of thyroid parameters

Serum TSH, FT3, FT4, TPOAb and TgAb were measured using an electrochemiluminescence immunoassay with Cobas Elecsys 601 (Roche Diagnostics), and quality control analyses were performed before, during and after testing according to the manufacturer's instructions. The reference ranges (0.27–4.20 IU/L for TSH, 3.1–6.8 pmol/L for FT3, 12–22 pmol/L for FT4, 0.00–34 IU/mL for TPOAb and 0.00–115 IU/mL for TgAb) were provided by the manufacturer. The intra-assay and inter-assay coefficients of variation (CVs) for TSH were 1.1–3.0% and 3.2–7.2%, for FT3 were 1.3–6.5% and 1.9–7.2%, for FT4 were 1.6–5.0% and 1.9–6.3%, for TPOAb were 2.7–6.3% and 4.2–9.5% and for TgAb were 1.3–4.9% and 2.1–6.3%.

Serum Sema 5A level

Serum Sema 5A levels were measured with ELISA kits (SEL924Hu) according to the manufacturer's instructions (CLOUD-CLONE CORP, Wuhan, China). The intra-assay CV value is less than 10% and the inter-assay CV value is less than 12%.

Preparation of RNA and quantitative real-time polymerase chain reaction (RT-PCR)

The PBMCs of all the participants were harvested, and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Then, cDNA was synthesized with a PrimeScript RT reagent kit (TaKaRa). The real-time PCR was carried out with a SYBR Premix Ex Taq kit (TaKaRa) using a LightCycler 480 RT-PCR system with the following conditions: 95°C for 30 s, then 95°C for 5 s and 60°C for 20 s with 45 cycles. The primer sequences for Sema 5A, plexin-A1, plexin-B3, IFN-γ, IL-4 and IL-17A are listed in Table 1. Gene expression is relative to the reference control (GAPDH).

Statistical analysis

Statistical analysis of the data was performed with SPSS software for Windows (version 22.0, SPSS Inc.). Normally distributed data are presented as the mean±standard deviation (M±s.d.), and the data with abnormal distributions are presented as the median (interquartile range) (IQR). Student's *t* test, one-way analysis of variance (ANOVA), non-parametric Mann–Whitney *U* tests or Chi-squared tests were used to analyse the differences among the groups depending on the characteristic of the data. Spearman correlation coefficients were used to determine correlations between the variables. Linear regression analyses were performed to detect the associations between thyroid parameters and serum Sema 5A adjusting for potential confounders (age, gender and BMI). ROC analysis was performed with MedCalc for Windows (version 15. 2, MedCalc Software, Ostend, Belgium). A *P* value <0.05 was considered statistically significant, and all tests were two-tailed.

Results

Clinical and demographic characteristics

The clinical and demographic features of the study are shown in Table 2. The age, gender and BMI in the healthy control group were similar to those in the HT group (all $P>0.05$).

There was no difference between the two groups in FT3 and FT4 (both $P>0.05$), but the TSH levels in the HT group (median: 3.33 IU/L, IQR: 2.30–5.03 IU/L) were significantly higher than those of healthy controls (median: vs 2.04 IU/L, IQR: 1.44–2.87 IU/L, $P<0.001$). The levels of TgAb and TPOAb in the HT group (median: 289 IU/mL, IQR: 141–492 IU/mL for TgAb; median: 209 IU/mL, IQR: 37.0–491 IU/mL for TPOAb) were significantly higher than those in healthy controls (median: 10.0 IU/mL, IQR: 10.0–10.0 IU/mL, $P<0.001$ for TgAb; median: 5.3 IU/mL, IQR: 5.0–8.2 IU/mL, $P<0.001$ for TPOAb).

Serum Sema 5A levels in patients with HT and healthy controls

The levels of serum Sema 5A in patients with HT (median: 1.31 ng/mL, IQR: 1.17–1.57 ng/mL) were significantly higher than that in the control group (median: 0.91 ng/mL, IQR: 0.88–0.94 ng/mL, $P<0.001$, Fig. 1). Further analysis revealed that serum Sema 5A levels both in eHT (median: 1.31 ng/mL, IQR: 1.03–1.51 ng/mL) and sHT (median: 1.30 ng/mL, IQR: 1.24–1.73 ng/mL) were significantly higher compared with that in the control group (both $P<0.001$), but no significant differences were detected between eHT and sHT ($P=0.425$). Moreover, serum Sema 5A levels were also positively associated with TgAb, TPOAb and TSH levels ($r=0.511$, $P<0.001$ for TgAb;

Table 1 Primers for semaphorin 5A, plexin-A1, plexin-B3, IL-17A, IFN-γ and GAPDH.

Gene		Sequence
semaphorin 5A	Forward	5′-TGGAAGACACCTGGACCACATTCA-3′
	Reverse	5′-ATCCAGCTCAGGCAGGAAGAAAGT-3′
plexin-A1	Forward	5′-CTCCCTGCCGTGGCTGCTCAACAA-3′
	Reverse	5′-ACCACAGTGCGGCCCCGATAGTCA-3′
plexin-B3	Forward	5′-AAAGCCACCGAGGAGCCAGAA-3′
	Reverse	5′-ACTTGACGGCGATGGGGATG-3′
IL-17A	Forward	5′-CAATCCCACGAAATCCAGGATG-3′
	Reverse	5′-GGTGGAGATTCCAAGGTGAGG-3′
IFN-γ	Forward	5′-TCGGTAACTGACTTGAATGTCCA-3′
	Reverse	5′-TCCTTTTTCGCTTCCCTGTTTT-3′
GAPDH	Forward	5′-GCACCGTCAAGGCTGAGAAC-3′
	Reverse	5′-TGGTGAAGACGCCAGTGGA-3′

Table 2 Clinical and demographic characteristics in patients with Hashimoto's thyroiditis and healthy controls.

Characteristics	Healthy controls (*n*=111)	Hashimoto's thyroiditis cases (*n*=92)	*P* values
Age, mean±s.d., years	45.2±6.8	45.6±8.2	0.702
Gender (female/male)	87/24	70/22	0.412
BMI, mean±s.d., kg/m^2	23.68±2.93	23.29±2.94	0.351
TSH, median, IU/L	2.04 (1.44–2.87)	3.33 (2.30–5.03)	<0.001
FT3, median, pmol/L	4.64 (4.27–4.91)	4.50 (4.09–4.78)	0.056
FT4, median, pmol/L	16.5 (15.1–17.8)	16.4 (15.7–18.1)	0.477
TgAb, median, IU/mL	10.0 (10.0–10.0)	289 (141–492)	<0.001
TPOAb, median, IU/mL	5.3 (5.0–8.2)	209 (37.0–491)	<0.001

BMI, body mass index; FT3, free T3; FT4, free T4; TgAb, antithyrogloblin antibody; TPOAb, thyroperoxidase antibody; TSH, thyroid-stimulating hormone.

r=0.423, *P*<0.001 for TPOAb; *r*=0.349, *P*<0.001 for TSH, Fig. 1). These relationships were also confirmed in linear regression analyses (*P*≤0.001) (Table 3). No relationship between serum Sema 5A and FT3 (*r*=−0.058, *P*=0.412) or FT4 (*r*=0.019, *P*=0.790) was found.

### The mRNA expression of Sema 5A and its receptors in HT patients

We further analyzed the RNA levels of Sema 5A and its receptors (plexin-B3 and plexin-A1) in PBMCs of HT patients. Consistent with serum Sema 5A levels, the mRNA expression of Sema 5A in the cases was significantly higher than that in the control group (*P*=0.041). The relative amount of plexin-B3 mRNA was increased significantly compared to that of controls (*P*=0.014), but no difference was found in the plexin-A1 mRNA expression between the groups (*P*=0.438, Fig. 2).

The mRNA expression of IFN-γ, IL-4 and IL-17

We also investigated the mRNA expression of IFN-γ, IL-4 and IL-17. The mRNA expression of IL-17 was significantly increased in the HT group (*P*<0.001). However, no difference was found between the groups in IFN-γ and IL-4 mRNA levels (*P*=0.497 and *P*=0.498, respectively). Spearman correlation analysis was performed to evaluate the association between serum Sema 5A and the mRNA expression of IFN-γ, IL-4 and IL-17. The results shown in Fig. 3 demonstrate that there was no correlation between serum Sema 5A and IFN-γ mRNA (*r*=−0.076, *P*=0.590) or IL-4 mRNA (*r*=0.074, *P*=0.604), but it was statistically correlated with IL-17 mRNA (*r*=0.442, *P*=0.001).

ROC analysis of Sema 5A

ROC analyses were performed, and the areas under the curve (AUCs) of the ROC curve were used to assess
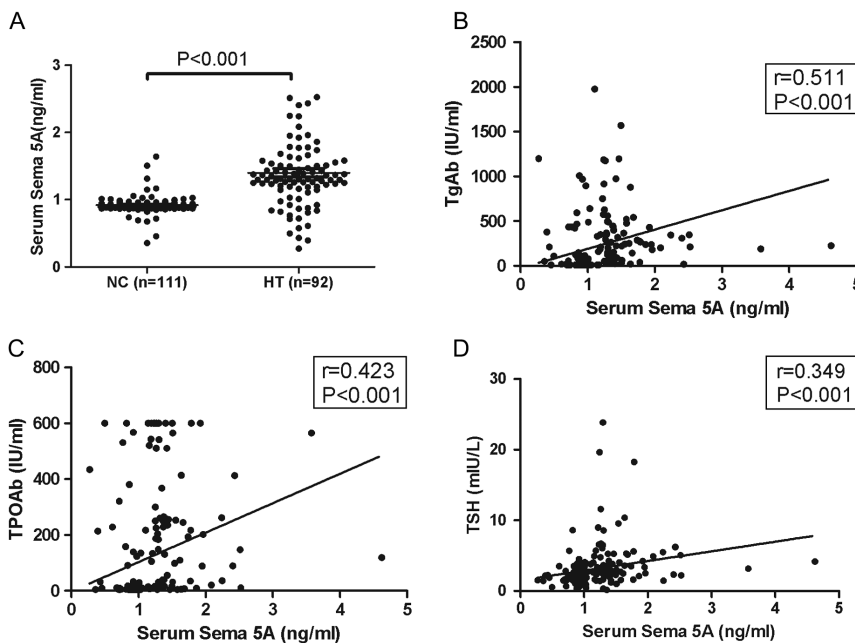
Figure 1
Serum semaphorin 5A (Sema 5A) levels in Hashimoto's thyroiditis patients (HT) and normal controls (NC). (A) Serum semaphorin 5A concentrations in HT were significantly higher than in healthy controls. Relationships among serum semaphorins 5A and TgAb, TPOAb and TSH (B, C and D).

Table 3 Linear regression analyses to analyse the associations between thyroid parameters (TSH, TPOAb and TgAb) and serum Sema 5A.

	Beta	95% CI	*P* value
TSH			
Age	0.006	−0.05, 0.06	0.819
Gender	0.424	−0.50, 1.34	0.365
BMI	0.001	−0.14, 0.14	0.994
Serum Sema 5A	1.337	0.55, 2.13	0.001
TgAb			
Age	−1.534	−10.69, 7.62	0.742
Gender	−94.097	−253.50, 65.30	0.246
BMI	12.990	−10.63, 36.61	0.279
Serum Sema 5A	227.858	90.93, 364.79	0.001
TPOAb			
Age	0.220	−3.32, 3.76	0.903
Gender	−5.667	−67.32, 55.98	0.856
BMI	−4.294	−13.43, 4.84	0.355
Serum Sema 5A	103.101	50.14, 156.06	0.000

BMI, body mass index; TSH, thyroid-stimulating hormone; TgAb, antithyroglobin antibody; TPOAb, thyroperoxidase antibody; Sema 5A, semaphorin 5A.

the risk of HT based on the serum Sema 5A levels (Fig. 4). The AUC of the serum Sema 5A was 0.836 (95% CI: 0.778–0.884, *P*<0.001) for all the participants. According to Youden's index, the best cut-off value for serum Sema 5A was 1.054 ng/mL. The sensitivity and specificity were 79.35% and 96.40%, respectively.

Discussion

In the present study, we reported for the first time the increased serum-soluble Sema 5A in patients with HT compared with healthy controls. Moreover, the elevated Sema 5A was correlated with IL-17 mRNA expression and thyroid auto-antibodies. Thus, Sema 5A may be involved in the immune response of HT patients.

Semaphorins were first identified as axon guidance molecules, and more than 20 types have been discovered to date (29). Increasing evidence has demonstrated the overlap and connection between nervous and immune systems (30). Several semaphorins, the so-called 'immune semaphorins', are essential in the regulation of immune system (31). Sema 4D, the first semaphorin found to have an immunomodulatory function (32) can activate B cells and dendritic cells (33). Sema 3A expressed in T cells was reported to inhibit T cell activation (34). In addition, a recent study demonstrated that Sema 7A strongly stimulated inflammatory responses of T cells and monocytes/macrophages in RA (21). However, whether Sema 5A is another immune semaphorin has not been demonstrated.

A recent study proposed the essential role of Sema 5A in innate immunity signaling pathways in dairy cows with mastitis (25). Subsequently, several studies revealed that Sema 5A potently modulates the production of cytokines and immune cell responses in autoimmune diseases, including RA (26), primary ITP (27) and SLE (28). We demonstrated elevated serum Sema 5A in HT, a type of autoimmune disease, which was consistent with these studies (21, 27, 28). Serum Sema 5A was positively correlated with TgAb levels and TPOAb (both *P*<0.001). Taken together, these results indicate an essential role of Sema 5A in immune responses of HT patients.

Evidence of increased serum Sema 5A in autoimmune disease has been revealed in RA (26), and recombinant soluble Sema 5A was shown to activate T cell proliferation and modulate cytokine production (including the Th17 cytokine IL-17A). Th17 cells and the cytokine IL-17A play essential roles in joint injury in RA patients (35). A previous study reported that Th17 cells were involved in the pathological process of HT (9). We did not measure serum IL-17A levels in our study, but the IL-17A mRNA expression in PBMCs from HT patients was elevated significantly. Moreover, a positive correlation between serum Sema 5A and IL-17A mRNA levels was also found in our patients; thus, further studies on the change of Th17 cells or inflammatory cytokines such as IL-17A, IL-4 and IFN-γ in HT patients and controls after recombinant Sema 5A stimulation may explore the exact role of Sema 5A on the differentiation of Th17 cells.
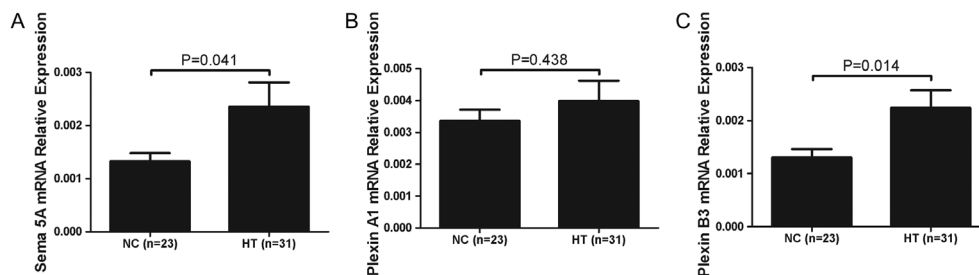
Figure 2
The mRNA expression of semaphorin 5A (Sema 5A, A), plexin-A1 (B) and plexin-B3 (C) in PBMCs of Hashimoto's thyroiditis patients (HT) and normal controls (NC).
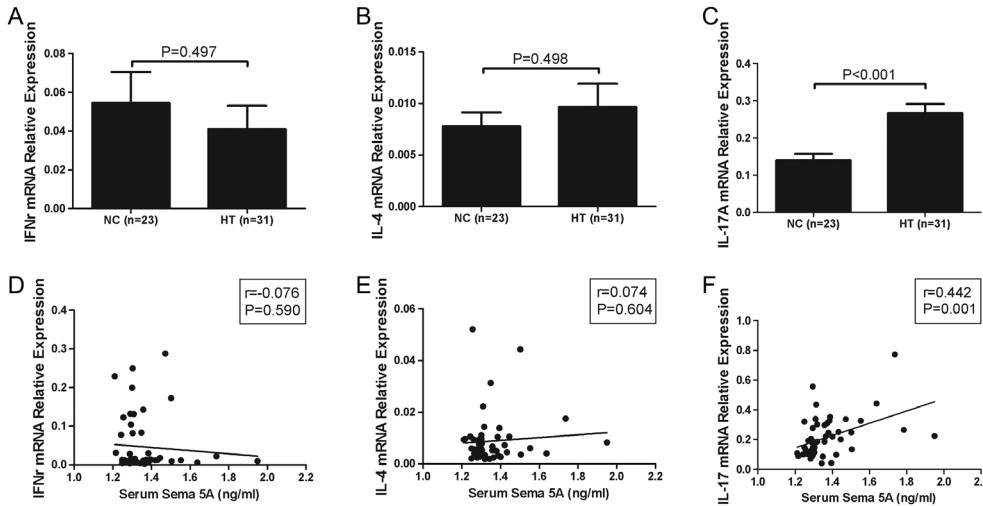
Figure 3

The mRNA expression of IFN-γ, IL-4 and IL-17A in PBMCs of Hashimoto's thyroiditis patients (HT) and normal controls (NC). RNA levels of IL-17A, but not IFN-γ and IL-4, in PBMCs of HT were significantly higher than in healthy controls (A, B and C). Serum semaphorin 5A levels were significantly correlated with IL-17A mRNA expressions (F), but not with IFN-γ and IL-4 (D and E).

Imbalance between Th1 cells and Th2 cells has long been thought to be the main nosogenesis of autoimmune diseases. But, in recent years, Th17 cells were identified as the critical mechanism of most autoimmune diseases, which could not be explained by the imbalance of Th1/Th2 (36). Li *et al.* reported that high dose of sodium iodide intake promoted more Th1 cells but moderate dose induced more Th17 cells in the mouse model of HT (9). In addition, previous studies have reported that patients with hypothyroiditism were characterized by higher proportion of Th1 cells and lower proportion of Th2 cells than patients with eHT (37). In this regard, Th1 cells possibly work in coordination with Th17 under different conditions of the disease. The mRNAs were from 23 HT patients with eHT in our study. Therefore, no difference was found between the groups in the mRNA expression of IFN-γ and IL-4 in our study, which may be explained by the condition of thyroiditis that was not severe enough in our cohort of patients.

Elevated serum Sema 5A can result from increased Sema 5A mRNA expression in PBMCs, as well as cleavage of membrane Sema 5A, which is dependent on ADAM17 (26, 28). ADAM17 is a member of the disintegrin and metalloprotease domain family, which has been shown to be involved in the cleavage of Sema 5A from cell membranes (38). In the present study, we detected an increased expression of Sema 5A in PBMCs of HT patients, but ADAM17 mRNA was not increased (data not shown). Thus, we hypothesized that elevated the Sema 5A mRNA expression in PBMCs may be the major source of soluble serum Sema 5A in HT.

To explore the possible mechanism of serum Sema 5A in HT, we also detected the expression of its receptors, plexin-A1 and plexin-B3 (39). These receptors were shown to have essential roles in the process of autoimmune diseases (26). We found elevated plexin-B3 but not plexin-A1 mRNA expression in PBMCs of HT patients. According to these data, we hypothesized that the activated Sema 5A/plexin-B3 pathway may participate in HT pathogenesis. Therefore, further research is needed to identify the exact mechanism of the Sema 5A/plexin-B3 pathway in HT.

In the present study, we also performed the ROC curve and showed the potential role of serum Sema 5A
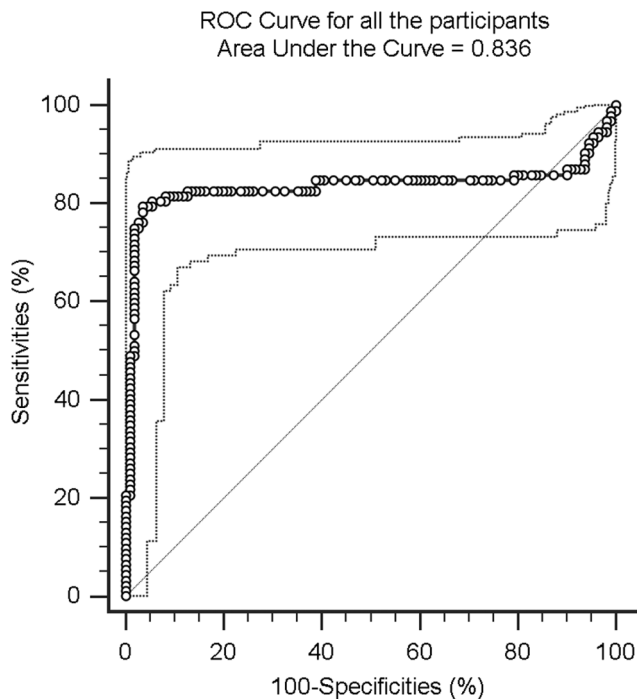

Figure 4

Receiver-operating characteristic (ROC) curves for predicting Hashimoto's thyroiditis by serum semaphorin 5A.

in the diagnosis of HT. With high sensitivity (79.35%) and specificity (96.40%), we propose that serum Sema 5A measurement may be useful in the diagnosis of HT and serum Sema 5A may be a complementary biomarker along with TPOAb and TgAb.

Our study also has some limitations. Because this is a single-center cross-sectional study, we failed to explore the dynamic changes of serum Sema 5A along with the treatment of HT. Moreover, we were unable to further elucidate the specific mechanism of Sema 5A in immune responses.

In conclusion, the present study demonstrated elevated serum Sema 5A in HT patients for the first time. The Sema 5A levels were correlated with thyroid auto-antibodies and may be involved in the immune response of HT patients. However, further research must be conducted to elucidate the mechanism of HT pathogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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