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Detection of the novel autoantibody (anti-UACA antibody) in patients with Graves' disease

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Abstract

Uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) is an autoantigen in patients with panuveitis such as Vogt–Koyanagi–Harada disease. The prevalence of IgG anti-UACA antibodies in patients with uveitis is significantly higher than healthy controls, suggesting its potential role as an autoantigen. Originally, UACA was cloned from dog thyroid tissue following TSH stimulation. So, we presumed UACA could be a novel autoantigen in autoimmune thyroid diseases. We measured serum anti-UACA antibody titer using ELISA in patients with autoimmune thyroid diseases (Graves' disease, Hashimoto's thyroiditis, subacute thyroiditis, and silent thyroiditis). The prevalence of anti-UACA antibodies in Graves' disease group was significantly higher than that in healthy group (15% vs. 0%). Moreover, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy was significantly higher than that in Graves' patients without ophthalmopathy (29% vs. 11%). Especially, 75% of severe ocular myopathy cases showed high UACA titer. Immunohistochemical analysis revealed that UACA protein is expressed in eye muscles as well as human thyroid follicular cells. Taken together, UACA is a novel candidate for eye muscle autoantigens in thyroid-associated ophthalmopathy.

Keywords: Uveal autoantigen with coiled coil domains and ankyrin repeats; Graves' disease; Graves' ophthalmopathy; Autoantigen; Ocular myopathy; FRTL5; Vogt–Koyanagi–Harada disease; Thyroid–eye shared autoantigen

Uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) is an autoantigen associated with panuveitis. Anti-UACA antibody appears in patients sera of Vogt–Koyanagi–Harada disease (VKH), sarcoidosis, and Behçet disease with uveitis. Although UACA is expressed in various tissues such as skeletal muscle and melanocyte, the appearance of anti-UACA antibody seems to reflect the autoimmune reaction against uveal melanocyte [1]. Interestingly, UACA was originally

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identified from dog thyroid as one of TSH regulated genes with unknown function [2]. The dual expression of UACA in thyroid and skeletal muscle led us to the idea that UACA could be an autoantigen associated with Graves' disease, since patients with Graves' disease frequently suffer from ophthalmopathy with ocular myopathy [3–15].

Thyroid-associated ophthalmopathy (TAO) is considered to be an autoimmune disorder of eye muscle and surrounding orbital connective tissue and fat, and the current dogma tells that TAO is induced by autoimmune reaction against thyroid and orbital tissue shared antigens [3–21]. One such candidate is TSH receptor, which is expressed in the orbital preadipocyte and fibroblast [8–11]. Several eye muscle and thyroid shared antigens also have clinical relevance in TAO; flavoprotein [16,17], 1D [18], and G2s protein [22–24]. The primary reaction in ocular tissue is thought to be T-cell-mediated autoimmunity against TSH receptor (TSHR) expressed in ocular fibroblasts. The appearance of antibodies against Fp, G2s, and 1D seems to be the secondary event in TAO process, reflecting the release of sequestered cytoskeletal proteins from damaged eye muscles [23,24]. These eye muscle proteins are expressed in eye muscles as well as skeletal muscles. Since UACA is expressed in skeletal muscle as well as thyroid tissue, we presumed that the appearance of anti-UACA autoantibody could be linked to the autoimmune response associated with Graves' ophthalmopathy.

In this study, we measured serum UACA antibody titer in patients with autoimmune thyroid diseases; healthy controls, Graves' disease, Hashimoto's disease, silent thyroiditis, and subacute thyroiditis. The mean value of UACA antibody titer in the Graves' disease group was significantly higher than healthy controls, but other group was not. Moreover, high UACA titer was observed in Graves' ophthalmopathy patients with severe ocular myopathy.

This is the first report describing the presence of anti-UACA autoantibodies in patients with Graves' disease. Especially, high UACA titer appears to be associated with eye muscle damage of Graves' ophthalmopathy.

Materials and methods

Study patients. We studied 159 Graves' disease, 26 Hashimoto's thyroiditis, 20 silent thyroiditis, 11 subacute thyroiditis, and 43 controls. We explained the purpose of this study to all subjects and obtained their informed consent. Graves' disease patients consisted of untreated 122 females and 37 males. Diagnosis of Graves' disease was confirmed by elevated free T_3 level (13.61 ± 6.67 ng/dl), undetectable TSH level, and positive TSH binding inhibitory immunoglobulin and/or thyroid stimulating antibody. They had hyperthyroidism symptoms such as palpitation and body weight loss. Silent thyroiditis group consisted of 18 females and 2 males with a mean age of 38 year. Diagnosis of silent thyroiditis was confirmed by elevation of free T_3 levels (7.51 ± 2.39 ng/dl), suppressed ^{123}I uptake, and elevated thyroglobulin level. Subacute thyroiditis patients had neck pain and tenderness. They had elevated free T_3 level (8.15 ± 5.48 ng/dl), CRP and ESR level, and suppressed TSH level. Hashimoto's thyroiditis group had elevated TSH levels (69.1 ± 38.2 μU/ml) and positive thyroid TGAb (antithyroglobulin antibody). Forty three normal individuals of similar age and gender were used as controls.

Graves' group included 31 patients with ophthalmopathy (24 females and 8 males, 21–58 years old), 128 patients without ophthalmopathy. The eye changes were classified according to an activity index (AI, 0–7) proposed by a committee of the International Thyroid Associations. Patients with ophthalmopathy were defined as >AI, and patients without ophthalmopathy were defined as A0. Ophthalmologic examination, including measurement of eye muscle function and performance of orbital MRI, was carried out on patients with ophthalmopathy. The congestive changes were defined as >AI, with or without eye muscle involvement. Ocular myopathy was defined as: diplopia and reduced eye movement associated with marked increase of eye muscle volume on orbital MRI. Congestive ophthalmopathy was defined as: nil or minimal eye muscle enlargement with, usually, a fibrotic appearance, as described by Ossoinig [25], features of periorbital inflammation (e.g., chemosis, lid swelling, and conjunctival injection), and no diplopia or reduced eye movements.

Human subjects. Human thyroid tissues were obtained by the University of Tottori committee for the protection of human subjects and in accordance with the Declaration of Helsinki. Thyroid tissue sample was obtained at surgery from a Graves' disease patient. Normal thyroid tissue was obtained at autopsy from a patient without thyroid disease. Human eye muscle tissue with Graves' ophthalmopathy was obtained at surgery from a Graves' disease patient (kindly provided by Dr. Yoichi Inoue, Olympia Eye Hospital).

Preparation of glutathione-S transferase fusion protein. A 783-bp DNA fragment digested from *Homo sapiens* cDNA clone IMAGE 608930 (Embank Accession No. AA197064) corresponding to nucleotide position 3462–4245 of UACA cDNA was inserted into pGEX4T-2 vector to produce glutathione-*S* transferase (GST) UACA fusion protein. This UACA fragment covers C-terminal 261 amino acids (18.0%) of whole UACA consisting of 1449 amino acids. Plasmids with this construct were transformed in *Escherichia coli* and incubated in 500 ml Luria broth medium for 8 h at 37 °C with shaking. Then, IPTG was added at a final concentration of 0.1 mM and the preparation was incubated for 16 h at 25 °C with shaking. This suspension was centrifuged and the pellet was suspended in 20 ml lysis buffer [50 mM Tris–HCl (pH 7.5), 25% sucrose]. Then, we added 100 μl of 10% Nonidet P-40, 1 M $MgCl_2$ on ice. The lysate was sonicated, centrifuged, and then the supernatant was incubated with 2 ml of slurry of glutathione–Sepharose 4B for 2 h at 4 °C. This suspension was centrifuged and the pellet was washed in WE buffer [20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, and 1 mM DTT] 10 times. The fusion protein was eluted with G buffer [5 mM GSH, 50 mM Tris–HCl (pH 9.6)] and eluted protein concentration was estimated by Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay. Detection and titration of antibody to a fragment of UACA were examined using indirect enzyme-linked immunosorbent assay (ELISA). GST–UACA fusion protein and GST protein were prepared and used as antigens. Microtiter plates (96-well) (NUNC, Denmark) were coated with GST–UACA fusion protein in PBS (pH 7.4) for 15 h at 4 °C. GST protein was simultaneously coated in different wells as control. The plates were then washed with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and incubated for 15 h at 4 °C with serum samples diluted at 1:50 with 1% skim milk/PBS. The plates were washed in PBS-T, and 100 μl of HRP-conjugated mouse anti-human IgG diluted at 1:2000 with 1% skim milk/PBS was added to each well followed by incubation at room temperature for 2 h. The plates were washed with PBS-T, and 100 μl solution of *o*-phenylenediamine (Sigma Fast; Sigma Chemical, St. Louis, MO) was added to each well. After

30 min, the reaction was stopped by adding 50 μl of 3 M H_2SO_4, and OD 490 nm was determined using a Model 550 microplate reader (Bio-Rad, Hercules, CA). The specific corrected OD value of an individual sample was calculated by subtracting the OD value of GST protein coated well from that of GST–UACA fusion protein.

Cell culture. FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD; ATCC No. CRL 8305) were a fresh subclone (F1) that had all properties previously detailed. All cells were grown in 6H medium consisting of Coon's modified F12 (Sigma Chemical, St. Louis, MO) supplemented with 5% calf serum, 1 mM non-essential amino acids (Gibco, Grand Island, NY), and a mixture of six hormones:bovine TSH (1×10^{-10} M), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Fresh medium was replaced every 2 or 3 days, and cells were passaged every 7–10 days. In different experiments, cells were maintained in 5H medium without TSH and then exposed to TSH for appropriate time period (0, 3, 6, 12, and 24 h). In dose course analysis, FRTL5 cells were incubated with various concentrations (0, 10^{-3}, 10^{-2}, 10^{-1}, and 1 mU/ml) of TSH for 24 h.

The following human thyroid cancer cell lines were obtained from Dr S. Kosugi (Department of Laboratory Medicine and Clinical Genetics Unit, Kyoto University School of Medicine); NPA [26] and FRO [27] thyroid cancer cell lines were grown in RPMI medium 1640 (31800-022, Gibco-BRL, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. FRO cells, derived from a poorly differentiated follicular thyroid carcinoma, were characterized by the presence of wild-type p53 alleles for exons 5–8 [24]. 8505C [28] and HTC [29] thyroid cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (12800-017, Gibco-BRL, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. Culture medium was changed every 2 days and cells were passaged every 5–6 days.

Western blot analysis. Cells were lysed on ice in 0.6 ml lysate mix containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride in PBS. For immunoblotting, 10 μg of each sample was electrically transferred to Immunobilon PVDF (polyvinylidene difluoride) Transfer Membranes (Millipore, Bedford, MA). Membranes were incubated in blocking buffer; Tris-buffered saline (TBS; Tris–HCl 10 mM, pH 8.0, and NaCl 150 mM) containing 0.05% [vol/vol] Tween 20 and 5% [wt/vol] non-fat dried milk for overnight. Membranes were then incubated in blocking buffer with rabbit polyclonal anti-UACA antibody (kindly provided by Dr. K. Yamada) (1:500 dilution) or goat polyclonal anti-actin antibody (sc-1616, Santa Cruz Biotechnology, USA) for 45 min, and then washed twice with TBS containing 0.05% Tween 20. Membranes were incubated in blocking buffer with horseradish peroxidase-conjugated anti-rabbit IgG antibody for UACA or horseradish peroxidase-conjugated anti-goat IgG antibody (Amersham, UK) for actin, respectively, washed three times with TBS with 0.05% Tween 20, and then detected with enhanced chemiluminescence reagents (Amersham, UK).

Reverse transcription-PCR. Poly(A)$^+$ RNA were purified from 10 μg of each total RNA and subjected to cDNA synthesis, using random primers and Superscript reverse transcriptase. Gene-specific PCR primers were designed to amplify fragments of 505 bp and used in the reverse transcription-PCR (RT-PCR) (94 °C 30 s, 56 °C 30 s, and 74 °C 4 min, 30 cycles). Forward and reverse primer sequences for PCR amplification of UACA were 5′-GAGAAAAGAAGTTGGAATCAT AA-3′ and 5′-TTGTGTAGGTGAGTTGGGAAAG-3′, respectively.

Immunohistochemical evaluation of UACA expression. UACA expression was analyzed by immunocytochemical staining of Graves' thyroid tissues, eye muscle tissue obtained from a patient with Graves' ophthalmopathy. We immunostained the ocular tissue including extraocular muscles. Paraffin-embedded tissue section, 4-μm thick, was deparaffinized in xylene, rehydrated through a graded alcohol series to deionized water. The endogenous peroxidase activity was blocked with H_2O_2. The tissue section was incubated with rabbit polyclonal anti-UACA antibody (1:10) for 12 h at 4 °C, then washed and incubated with biotinylated horse anti-rabbit IgG (1:3000) for 30 min at room temperature. The sections were immersed in a solution with the avidin–biotin complex (Vector Laboratories, USA) for 30 min, developed with diaminobenzidine, and counterstained with eosin. The sections were scanned at magnification (200×, 400×) using light microscopy. Normal thyroid sample was obtained at autopsy from a patient without thyroid disease.

Immunofluorescent staining and microscopy. FRTL5 cells were plated on coverslips and cultured in Coon's modification HamF 12 with 5% fetal calf serum, then washed twice with PBS, and fixed with 2% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100, incubated with rabbit polyclonal anti-UACA antibody (1:100), and then visualized using FITC-conjugated anti-rabbit IgG antibody. In order to observe the fine localization of UACA protein within cells, we used confocal microscopy system (FLUOVIEW-OLYMPUS).

Statistical analysis. We used the χ^2 test (with Yeasts' correction for small numbers) and Fisher's exact test for categorical comparisons of the data. Differences in the means of continuous measurements were statistically analyzed using ANOVA. P value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed on a personal computer with the statistical package StatView 5.0 for Macintosh (SAS Institute, Cary, NC).

Results

ELISA

We measured serum UACA antibody titer in patients with autoimmune thyroid diseases in ELISA, using recombinant C-terminal 18% fragment of human UACA protein. We measured titer of healthy controls (43 cases), Graves' disease (159 cases), Hashimoto's disease (26 cases), silent thyroiditis (20 cases), and subacute thyroiditis (11 cases). To exclude the effect of reactivity against GST protein, we used GST–UACA fusion protein and GST protein for ELISA, simultaneously. Evaluation of IgG anti-UACA autoantibodies was determined by subtracting the reactivities against GST from those against GST–UACA. The mean OD value of anti-UACA autoantibodies in Graves' patients was significantly higher than that in healthy controls (ANOVA; $P < 0.01$) (Fig. 1A). This group patient did not accompany VKH disease or other uveitis. In contrast, Hashimoto's thyroiditis, silent thyroiditis, and subacute thyroiditis group did not show any statistical significance compared with control. The cutoff OD value for positivity of anti-UACA IgG antibodies was defined as the mean value +3 SD of healthy controls (0.53). We found anti-UACA IgG antibodies in 15% (24/159) of Graves' patients and 0% (0/43) of healthy control (Table 1). The prevalence of IgG anti-UACA antibodies in Graves' patients was significantly higher than that in healthy control (Fisher's exact test; $P < 0.05$). Anti-UACA antibodies were found in 4% (1/26) of Hashimoto's thyroiditis, 5% (1/20) of silent thyroiditis, and 8% (1/11) of subacute thyroiditis group. The differences in prevalence of anti-UACA antibodies

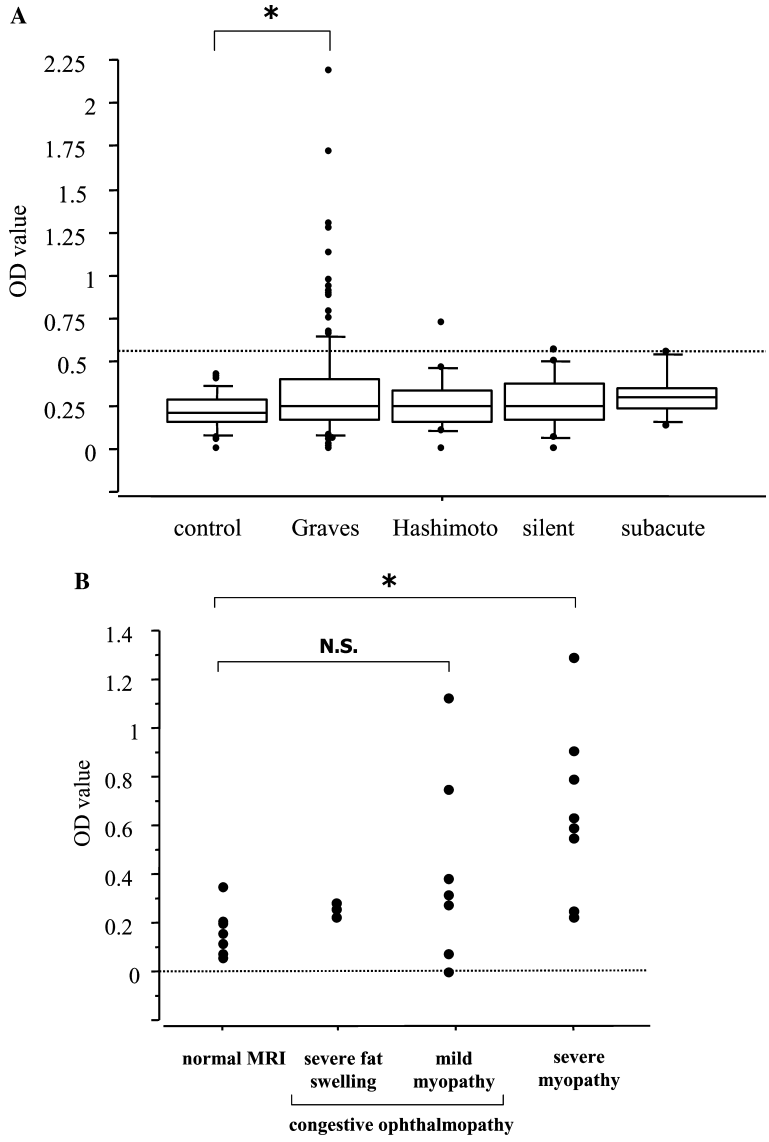

Fig. 1. (A) Distribution of anti-UACA IgG autoantibodies titer in healthy controls (control), in patients with Graves' disease (Graves), Hashimoto's disease (Hashimoto), silent thyroiditis (silent), and subacute thyroiditis (subacute). The titers of autoantibodies are expressed in the OD units. Broken line indicates a cutoff level for the positivity of autoantibody. The OD value subtracted GST protein from GST–UACA fusion protein in 159 Graves' disease samples was 0.339 ± 0.305 (mean ± SE), in 26 Hashimoto's thyroiditis samples was 0.269 ± 0.151 (mean ± SE), in 20 silent thyroiditis samples was 0.269 ± 0.156 (mean ± SE), in 11 subacute thyroiditis samples was 0.315 ± 0.130 (mean ± SE), and in 43 healthy control samples was 0.218 ± 0.103 (mean ± SE). The OD value for GST–UACA fusion protein to GST protein in 27 positive samples was 0.865 ± 0.384 (mean ± SE), and in negative samples was 0.240 ± 0.125 (mean ± SE). *Significant difference ($P < 0.01$) compared with control (ANOVA). (B) Distribution of anti-UACA IgG autoantibodies titer in patients with Graves' ophthalmopathy. The clinical manifestations of Graves' ophthalmopathy are classified into the following four groups; normal MRI, congestive ophthalmopathy with severe fat swelling, congestive ophthalmopathy with mild myopathy, and severe myopathy. The OD value subtracted GST protein from GST–UACA fusion protein in seven normal MRI samples was 0.167 ± 0.098 (mean ± SE), in three congestive ophthalmopathy with severe fat swelling samples was 0.255 ± 0.029 (mean ± SE), in seven congestive ophthalmopathy with mild myopathy samples was 0.418 ± 0.395 (mean ± SE), and in eight severe myopathy samples was 0.656 ± 0.349 (mean ± SE). *Significant difference ($P < 0.005$) compared with normal MRI (ANOVA). NS: not significant.

were not statistically significant between Hashimoto's thyroiditis and healthy control, silent thyroiditis and healthy control, and subacute thyroiditis and healthy control. More than half of Graves' patients with positive titer showed higher titer than patients with VKH disease. Positive patient sera with VKH disease showed about 0.5 OD value in the same ELISA.

Clinical manifestation

We investigated clinical manifestation of Graves' patients with high anti-UACA titer in detail. We found 37.5% (9/24) cases had ophthalmopathy. Especially, patient samples with severe eye muscle inflammation showed high anti-UACA titer. Nine cases with Graves'

Table 1
Prevalence of IgG anti-UACA autoantibodies evaluated by ELISA in sera from patients with thyroid diseases and healthy controls

Disease	Anti-human UACA IgG positive donors		P value
Graves' disease	24/159	15%	$P < 0.01$
Hashimoto's disease	1/26	4%	
Silent thyroiditis	1/20	5%	
Subacute thyroiditis	1/11	9%	
Healthy controls	0/43	0%	

Differences in prevalence of IgG anti-UACA autoantibodies are statistically significant between patients with Graves' disease and healthy controls using Fisher's exact test (2 × 2 table).

ophthalmopathy showed high titer (OD value >0.53) within 31 Graves' ophthalmopathy cases. The prevalence of anti-UACA antibodies was 29% in Graves' ophthalmopathy cases. But, the prevalence of anti-UACA antibodies in patients without ophthalmopathy was 11% (15/128). The prevalence of Graves' ophthalmopathy cases was significantly higher than that of Graves' patients without ophthalmopathy (Fisher's exact test; $P < 0.05$) (Table 2). Within nine cases of high titer, six cases showed severe eye muscle inflammation in MRI study. Graves' ophthalmopathy is classified into the following four groups; severe ocular myopathy, congestive ophthalmopathy with mild myopathy, congestive ophthalmopathy with severe orbital fat swelling and without myopathy, and normal MRI. The prevalence of anti-UACA antibodies was 75% (6/8) in severe ocular myopathy, which had severe eye muscle enlargement and high intensity signal within eye muscle in T2WI MRI study. In contrast, the prevalence of anti-UACA antibodies was 28% (2/7) in congestive ophthalmopathy cases with mild myopathy, who had mild eye muscle enlargement and high intensity signal in T2WI MRI study. The prevalence of anti-UACA antibodies was 0% (0/3) in congestive ophthalmopathy case, who had severe orbital fat swelling, no eye muscle enlargement, and no high intensity in T2WI MRI study. UACA titer of seven patients with normal MRI study was all normal.

The mean UACA titer of severe ocular myopathy cases was significantly higher than that of seven normal cases in MRI study (ANOVA; $P < 0.005$) (Fig. 1B). The prevalence of anti-UACA antibodies was 75% (6/8) in patients with severe ocular myopathy. The prevalence was significantly higher than that of normal MRI group (Fisher's exact test; $P < 0.01$) (Table 3). But, any other group did not show the significant difference compared with normal MRI group.

Table 2
Prevalence of IgG anti-UACA autoantibodies evaluated by ELISA in sera from patients with Graves' ophthalmopathy and without Graves' ophthalmopathy

Group	Anti-human UACA IgG positive donors		P value
Graves' ophthalmopathy (+)	9/31	29%	$P < 0.05$
Graves' ophthalmopathy (−)	15/128	11%	

Statistical analyses refer to differences between patients with Graves' ophthalmopathy and without Graves' ophthalmopathy determined using χ^2 test (2 × 2 table, Yeasts' correction for small numbers).

Table 3
Prevalence of IgG anti-UACA autoantibodies evaluated by ELISA in sera from patients with Graves' ophthalmopathy and MRI study

Group	Anti-human UACA IgG positive donors		P value
Severe ocular myopathy	6/8	75%	$P < 0.01$
Congestive ophthalmopathy (with mild myopathy)	2/7	28%	
Congestive ophthalmopathy (with severe orbital fat swelling)	0/3	0%	
Normal MRI	0/7	0%	

Statistical analyses refer to differences between patients with severe ocular myopathy and Normal MRI group determined using Fisher's exact test (2 × 2 table).

Expression of UACA in thyroid

To examine mRNA expression of UACA in thyroid, we performed RT-PCR analysis (Fig. 2C). Gene-specific PCR primers were designed to amplify 505 bp fragments of C-terminal portion of human UACA cDNA. The expression of UACA mRNA was observed in all human thyroid cancer cell lines (HTC, 8505C, FRO, and NPA), as well as human thyroid tissue of Graves' disease, Hashimoto's thyroiditis, and normal control. This result indicates UACA mRNA is expressed in human thyroid follicular cells.

To examine the expression profile of UACA protein in FRTL5 cell, we performed Western blot analysis. UACA encoded 160 kDa protein (Figs. 2A and B). The amount of UACA protein was augmented in a time (0, 3, 6, 12, and 24 h) (Fig. 2A) and dose-dependent manner following TSH stimulation (0, 10^{-3}, 10^{-2}, 10^{-1}, and 1 mU/ml) (Fig. 2B). In a time course, UACA protein increased after 3 h following TSH stimulation. The strongest signal was observed after 6 or 12 h following TSH stimulation, and the signal decreased after 24 h following TSH stimulation. In a dose course of TSH, the minimum concentration of TSH to increase UACA protein was 10^{-3} mU/ml.

In order to study the cytochemical localization of UACA, we estimated the expression of UACA in FRTL5 cells. The UACA protein was weakly expressed both in nucleus and cytoplasm of cells in the absence of TSH (Fig. 3A). Interestingly, TSH stimulation recruited UACA into nucleus (Fig. 3A; 24 h). In order to observe the fine localization of UACA within TSH-stimulated

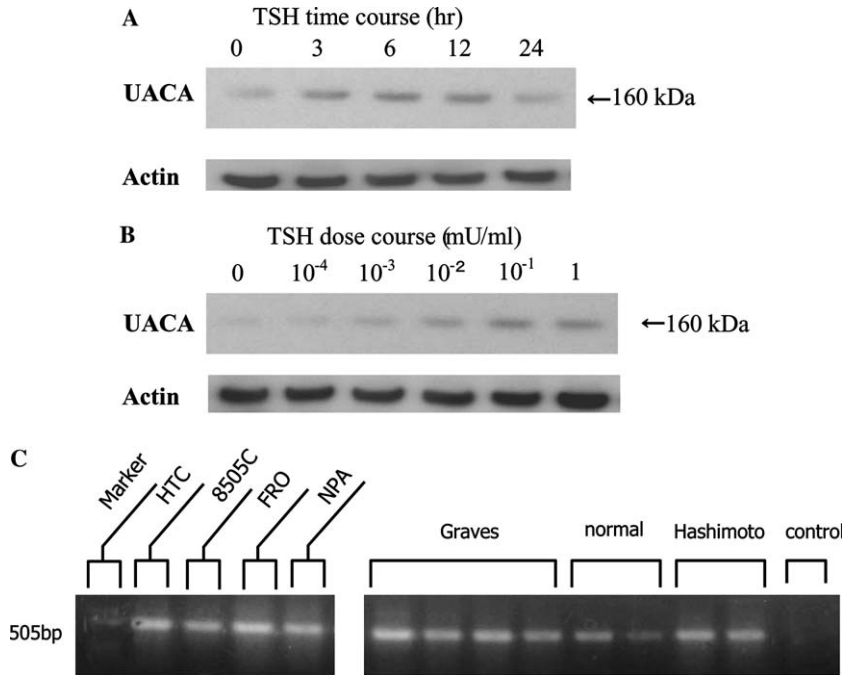

Fig. 2. TSH potentiates UACA protein expression in FRTL5 thyroid cells: (A) time sequence, (B) dose dependency. FRTL5 cells were preincubated in 5H medium with 5% CS for 5–6 days and then incubated with appropriate concentrations of TSH. To ensure the total amount of protein in each lane was identical, membranes were simultaneously incubated with anti-actin antibody (1:250 dilution). (C) RT-PCR analysis using human UACA-specific primers revealed the expression of UACA mRNA in human thyroid cancer cell lines (HTC, 8505C, FRO, and NPA), and the thyroid tissue of Grave's disease, Hashimoto's disease, and normal subject. Control lane indicates PCR product when any template was not included in PCR.

FRTL5, we used a differential interference contrast image. Most of the UACA fluorescence was localized within nucleus, whereas less was localized in cytoplasm (Fig. 3B). To examine UACA expression in human thyroid tissue, we then carried out an immunohistochemical analysis using rabbit polyclonal anti-UACA antibody. In thyroid tissue of Graves' disease, UACA appeared to be expressed in the nucleus of thyroid follicular cells (Fig. 3C).

Expression of UACA protein in eye muscle

In order to investigate the association of Graves' ophthalmopathy and anti-UACA antibody production, we examined UACA expression in human eye muscle derived from a patient with Graves' ophthalmopathy. In human eye muscle tissue with Graves' ophthalmopathy, UACA protein was exclusively expressed in eye muscle fiber (arrow), but UACA expression was relatively weak in surrounding orbital connective tissue and fat (Fig. 3D). The eye muscle sample was derived from a patient with Graves' ophthalmopathy, who was already treated by methimazole and corticosteroid. Since this pretreatment may modify the UACA expression in eye muscle, we simultaneously examined UACA expression in normal rat eye muscle. UACA protein was expressed in normal rat eye muscle fiber as observed in the human sample (data not shown). This result indicates UACA is expressed in eye muscle fiber as well as thyroid cells, which are the autoimmune target tissues in Graves' disease.

Discussion

UACA is a protein cloned by serological analysis of recombinant cDNA expression libraries (SEREX) method with serum samples obtained from patients with VKH disease, to identify the target autoantigens in VKH disease [1]. VKH disease is recognized as an autoimmune systemic disorder. In VKH, inflammatory disorders in multiple organs include melanocytes, uvea (resulting in acute bilateral panuveitis), skin (vitiligo and alopecia), central nervous system (meningitis), and inner ears (hearing loss and tinnitus). These inflammatory aspects are attributed to the immunological destruction of melanocytes. The prevalence of IgG anti-UACA autoantibodies is 19.6% in patients with VKH, and 0% in the healthy controls, 28.1% in patients with Behçet disease, and 21.1% in patients with sarcoidosis, so anti-UACA autoantibodies are considered as one of the autoantibodies in these panuveitis diseases. Originally, UACA was cloned from dog thyroid tissue following TSH stimulation, so we presumed UACA could be a novel candidate of autoantigen in autoimmune thyroid diseases [2]. Then, we analyzed the

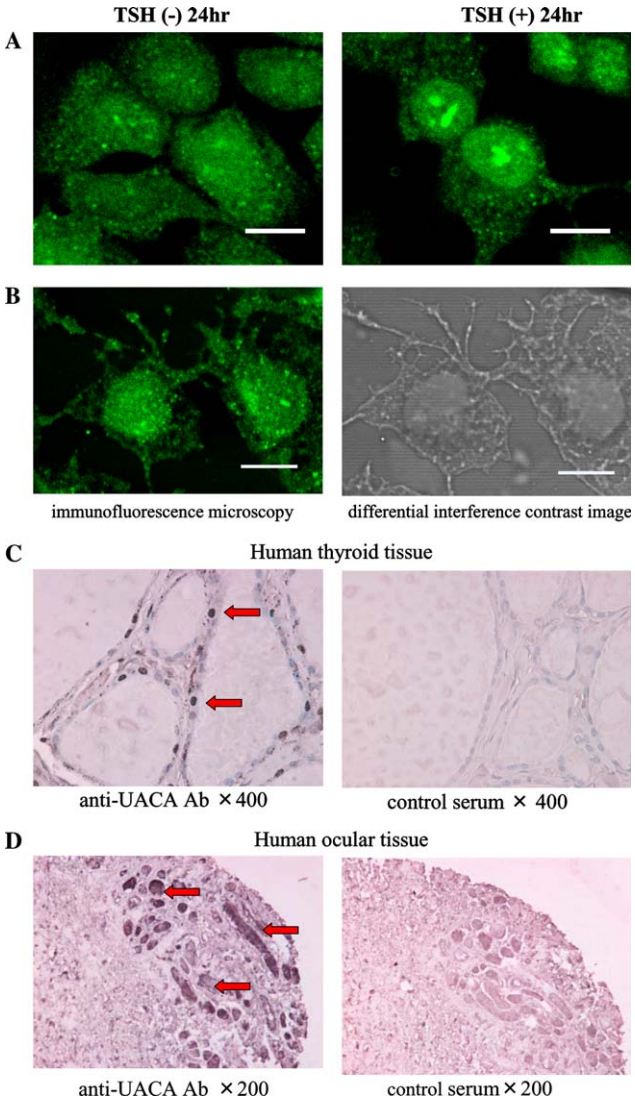

Fig. 3. (A) The cytochemical localization of UACA protein in FRTL5 cells in the absence or presence of TSH (1 mU/ml, 24 h). Inserted bar indicates 10 μm. (B) The fine cytochemical localization of UACA protein in FRTL5 cells followed by TSH stimulation (TSH 1 mU/ml, 24 h). Differential interference contrast image (right panel), and immunofluorescence microscopy image of FRTL5 cells (left panel). Inserted bar indicates10 μm. (C) The expression of UACA protein in human thyroid tissue of Graves' disease. Immunohistochemical analysis was done using rabbit anti-UACA antibodies. The sections were scanned at magnification (400×) using light microscopy. The arrows indicate nucleus of thyroid follicular cell expressing UACA protein. (D) The expression of UACA protein in human eye muscle tissue with Graves' ophthalmopathy. The sections were scanned at magnification (200×) using light microscopy. The arrows indicate eye muscle fibers expressing UACA proteins.

presence of anti-UACA antibodies in autoimmune thyroid diseases.

In ELISA study, the prevalence of anti-UACA antibodies in Graves' disease was significantly higher than that in healthy controls (15% vs. 0%). Moreover, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy was significantly higher than that in Graves' disease without ophthalmopathy (29% vs. 11%). We then investigated clinical manifestation of these patients with high UACA titer in detail. The prevalence of anti-UACA antibodies was 29% (9/31) in Graves' ophthalmopathy cases. These results clearly suggest that the appearance of anti-UACA antibody is strongly associated with eye muscle inflammation in patients with Graves' ophthalmopathy.

Thyroid-associated ophthalmopathy is considered to be an autoimmune disorder of eye muscle and surrounding orbital connective tissue and fat [3–7]. The eye symptoms associated with TAO can be classified into two subtypes, congestive ophthalmopathy (CO), in which inflammatory changes in periorbital tissues predominate, and ocular myopathy (OM), in which eye muscle is mainly damaged [30]. The current dogma tells that TAO is best explained by reactivity against thyroid and orbital tissue shared autoantigens [6,7]. One of such shared antigens is TSH receptor (TSHR), which is expressed in orbital preadipocytes. TSHR is mainly associated with the development of Graves' ophthalmopathy [8–11]. Several shared eye muscle and thyroid autoantigens have been investigated in eye muscle component in TAO, such as 63–67 kDa eye muscle membrane antigens and 55 kDa protein [12–24]. The flavoprotein (Fp) subunit of the mitochondrial enzyme succinate dehydrogenase is the so-called 64-kDa protein. Antibodies against Fp seem to be the best clinical marker of ophthalmopathy in patients with Graves' hyperthyroidism, and they are sensitive predictors for the development of eye muscle dysfunction in ophthalmopathy patients treated by antithyroid drugs [16,17]. The "55-kDa protein" was identified as G2s protein, eye muscle shared autoantigen with unknown function [22–24]. The primary reaction in eye muscle may be T-cell-mediated autoimmunity against TSHR of fibroblasts. The antibodies against Fp and G2s are produced secondary during the ophthalmopathy process, reflecting the release of sequestered cytoskeletal proteins from damaged eye muscles. Our observation indicates that UACA could be a novel candidate for thyroid and orbital shared autoantigen such as Fp in Graves' ophthalmopathy.

We also showed that UACA is expressed in eye muscle of patients with TAO as well as thyroid follicular cells in Graves' disease by immunohistochemical analysis. UACA was highly expressed in human eye muscle fibers of Graves' disease (Fig. 3D). This result indicates that UACA is simultaneously expressed in orbital eye muscle as well as thyroid follicular cells. High prevalence of anti-UACA antibodies is observed in patients with Graves' ophthalmopathy (Fig. 1). In particular, patients with severe ocular myopathy showed high UACA titer. Taken together, we presume that the appearance of anti-UACA antibodies could be a clinical marker for severe ocular myopathy, especially when its titer is high.

Wall and co-workers [16,17] suggested that anti-flavoprotein antibodies are produced by secondary immunoregulatory event resulting from eye muscle necrosis. They also showed that the prevalence of anti-G2s antibodies was 50% in Graves' ophthalmopathy, and anti-G2s antibodies appear in early phase of TAO [22–24]. In our study, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy is lower (29%) compared with anti-G2s antibodies. If UACA is the primary autoantigen in Graves' ophthalmopathy, the prevalence should be higher. It is more likely that anti-UACA Ab is produced by secondary immunoregulatory process resulting from eye muscle necrosis like anti-flavoprotein antibodies. The low prevalence of anti-UACA Ab may raise the possibility that UACA is not relevant for the development of TAO. But, the prevalence of anti-UACA antibodies is only 20% in VKH disease, the original disease with anti-UACA antibodies. Because of the difficulty to produce recombinant UACA protein as a whole molecule (160 kDa), we used the C-terminal 18.0% portion of UACA to detect anti-UACA antibodies in patients' sera. A relatively lower prevalence of anti-UACA antibodies in Graves' patients may be due to the limited usage of C-terminal fragment of UACA protein for ELISA. To evaluate the presence of autoantibody against the whole UACA molecule, it is necessary to analyze patients' sera using other N-terminal fragments of UACA protein.

At present, the recognition of TSHR on the retro-ocular preadipocytes by TSHR autoantibodies and TSHR-specific T cells could be the initial event that drives the "homing" of the lymphocytes to the retro-orbital tissue. Then, the eye muscle inflammation is activated, resulting in the appearance of eye muscle autoantibodies including G2s, Fp, and UACA. Consequently, our observation about anti-UACA Ab is not contrary to this theory explaining the development of TAO. Since UACA is expressed in eye muscle, the appearance of anti-UACA antibodies may reflect immunological damage of eye muscle fiber, as observed in flavoprotein. We presume that not thyroid destruction but eye muscle destruction is directly associated with the production of anti-UACA antibody. If we examine more TAO cases with anti-UACA antibodies, we can identify the clinical relevance of anti-UACA antibodies for the development of TAO.

Although the physiological function of UACA protein is still unclear, UACA contains six ankyrin repeats and coiled coil domains, including a motif of leucine zipper pattern. Ankyrin repeat is 31–33 amino acid motif present in a number of proteins and contributing to protein–protein interactions [31]. In FRTL5 thyroid cells, the amount of UACA protein increased in a time- and dose-dependent manner following TSH stimulation. In the absence of TSH, UACA protein was diffusely distributed both in nucleus and cytoplasm of FRTL5 cells. Following TSH stimulation, UACA protein was exclusively recruited into nucleus of FRTL5 cell (Fig. 3A). Consequently, TSH augments UACA expression and simultaneously converts the localization of UACA within FRTL5 thyroid cells. Interestingly, UACA protein was highly expressed in nucleus of thyroid follicular cell in human thyroid tissue of Graves' disease. These results suggest that UACA protein may play a potential role for thyroid cell proliferation, since TSH drives the growth of thyroid follicular cells. Further study is necessary to reveal the physiological relevance of UACA in thyroid cell proliferation.

In summary, we demonstrate the high prevalence of anti-UACA autoantibodies in patients with Graves' disease. We confirmed that patients with Graves' ophthalmopathy (especially, with severe ocular myopathy) showed high UACA titer. UACA protein is expressed in autoimmune target tissues of Graves' disease, such as thyroid follicular cells and ocular eye muscles, indicating UACA is a novel thyroid–eye shared autoantigen. Although the sequence of autoantibodies production such as anti-G2s, anti-Fp or anti-UACA remains unknown, anti-UACA antibodies could be a clinical marker of ocular myopathy in patients with Graves' ophthalmopathy.

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