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ORIGINAL ARTICLE

Effect of antithyroid drugs on the occurrence of antibodies against type 2 deiodinase (DIO2), which are involved in hyperthyroid Graves' disease influencing the therapeutic efficacy

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Abstract

Graves' disease is an organ-specific autoimmune disease with hyperthyroidism, diffuse goiter and autoantibodies against TSH receptor, thyroid peroxidase (TPO) and/or thyroglobulin (Tg). Graves' hyperthyroidism is characterized by T_3 dominance due to the conversion of T_4 into T_3 through type 1 and 2 deiodinase enzymes (DIO1, DIO2). Methimazole (MMI) and propylthiouracil (PTU) therapies inhibit thyroid hormone synthesis blocking the activity of deiodinase and TPO enzymes. The study investigated the occurrence of autoantibodies against DIO2 peptides (cys- and hom-peptides) with the effect of antithyroid drugs on their frequencies in 78 patients with Graves' disease and 30 controls. In hyperthyroidism, the presence of DIO2 peptide antibodies was as follows: 20 and 11 cases out of 51 for cys- and hom-peptide antibodies, respectively, of whom 8 cases possessed antibodies against both peptides. Antithyroid drugs differently influenced their frequencies, which were greater in PTU than in MMI (3/6 vs 13/45 cases, $P < 0.016$ for cys- and 0/6 vs 2/45 cases for hom-peptide antibodies). Antibodies against both peptides demonstrated more reduced levels of anti-TPO ($P < 0.003$) and anti-Tg antibodies ($P < 0.002$) compared with those without peptide antibodies. PTU compared with MMI increased the levels of TSH receptor antibodies (32.5 UI/l vs 2.68 IU/l, $P < 0.009$). MMI treatment led to more reduced FT_3 levels and FT_3/FT_4 ratios in hyperthyroid Graves' ophthalmopathy ($P < 0.028$ for FT_3, $P < 0.007$ for FT_3/FT_4 ratio). In conclusion, the presence of DIO2 peptide antibodies is connected to Graves' hyperthyroidism influencing the levels of antibodies against TPO, Tg and TSH receptor, as well as the therapeutic efficacy of antithyroid drugs.

Keywords Antithyroid drugs · Hyperthyroidism · Graves' disease · Antithyroid antibodies · Type 2 deiodinase

Introduction

Graves' disease is a member of autoimmune thyroid disease (AITD) characterized by hyperthyroidism, diffuse goiter and antibodies mainly against TSH receptor, thyroid peroxidase (TPO) and/or thyroglobulin (Tg) [1]. TSH receptor antibodies play a crucial role in the development of hyperthyroidism [2]. The causes of antithyroid antibody productions are considered as a breaking of the central and peripheral tolerance against thyroidal antigens due to genetic, environmental, stress or other unknown factors [3]. The increase in thyroid hormone synthesis is associated with the onset of Graves' disease showing T_3 dominance [4]. Type 1 (DIO1) and type 2 (DIO2) deiodinase enzymes convert T_4 into T_3, of those DIO1 plays the main role in Graves' hyperthyroidism [5]. DIO2 deiodinase is a common enzyme in thyroid and eye muscle tissues highlighting its potential role in the thyroid-associated ophthalmopathy (later called Graves' ophthalmopathy) [6].

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Antithyroid drugs, methimazole (MMI) and propylthiouracil (PTU), are the main, first-choice medicines for inhibition of thyroid hormone synthesis in Hungary. The antithyroid drugs bound to DIO and TPO enzymes blocked the conversion T_4 into T_3 and lead to the inhibition of TPO effect on iodination and the phenolic coupling of iodotyrosine residues (production of T_4) [7]. Antithyroid drugs, particularly PTU, can induce antineutrophil cytoplasmic antibodies (ANCAs), but very rarely associated with manifest vasculitis [8].

In this study, the occurrence of antibodies directed to DIO2 peptides was investigated in the presence and absence of treatment with antithyroid drugs, as well as their therapeutic efficacy on thyroid hormone levels.

Patients and methods

Patients

One hundred and eight patients were investigated, of whom 78 had Graves' disease [median of age: 46.5 years and interquartile range (IQR): 29–53.5 years, 20 males, 38 had ophthalmopathy] and 30 controls (median age: 50.5 years and IQR: 35.75–56.25 years, 3 males). The diagnosis of Graves' disease was based on the presence of hyperthyroidism and diffuse goiter with or without eye symptoms. Radioscintigraphy with technetium^{99} and ultrasonography were used for the exclusion of toxic adenoma, where hyperthyroidism was associated with nodular goiter. Fifty-one patients were hyperthyroid, 24 patients euthyroid and 3 patients hypothyroid at the time of the study. The duration of Graves' disease was given in median: 7 months and IQR: 2–11 months. The diagnosis of ophthalmopathy was based on NOSPECS with ophthalmic control [9]. Soft tissue involvement and proptosis were found together in the patients with ophthalmopathy ($n = 37$), of whom 18 patients had diplopia due to eye muscle enlargements. None of the patients showed corneal ulceration or optic nerve damage. Sixteen patients had the clinical activity scores for ophthalmopathy, CAS ≤ 4 and 23 cases above 4. The median of CAS was 5 and IQR: 4–7 [10]. Twenty-seven cases have not been treated yet; in 51 cases, the patients received antithyroid drugs (6 cases PTU and 45 cases MMI). Thirty healthy persons formed the control group.

Methods

The measurement of antibodies against DIO2 peptides

Sandwich enzyme-linked immunosorbent assay (ELISA) was used for the detection of antibodies against two DIO2 peptides (cys- and hom-peptides). The amino acid (aa) sequences of DIO2 peptides were corresponded to DIO2 (GenBank AAD45494-1) aa sequences between 124 and 152 containing the active center of enzyme: ^{124}leu-val-val-asn-phe-gly-ser-ala-thr-^{133}**cys**-pro-pro-phe-thr-ser-glu-leu-pro-ala-phe-arg^{144} for **hom-peptide** and ^{132}thr-^{133}**cyscys**-pro-pro-*^{136}thr*-*^{137}phe*-ser-glu-leu-pro-ala-phe-arg-lys-leu-val-glu-glu-phe-ser-ser^{152} for **cys-peptide** (replaced double cyscys at position 133 and aa reversed at positions 136 and 137). Guinea pigs were immunized with cys-peptide to produce capture antibodies, which were tested before with antibody titration using serial dilutions. The procedures of peptide synthesis and the guinea pig immunization were described in detail in our previous paper [11].

The 96-well plates (Dynatech Immunolon™, USA) were covered with 100 µl/well guinea pig antibodies against cys-peptide in dilution of 1:500 using carbonate buffer (pH 9.6) at 4 °C for overnight. The plates were washed 3 times with Tween PBS (phosphate-buffered saline, pH 7.4, 0.05% Tween 20, Reanal, Hungary). Bovine serum albumin in 1% solution (BSA, SIGMA, USA) was applied for blocking the nonspecific binding at 37 °C for 1 h. When the plates had been washed three times, the DIO2 peptides (cys- or hom-peptides) were added to the plates in the concentration of 0.3 µg/100 µl/well at room temperature for 2 h. In the end, the plates were washed, and 100 µl/well patient sera in dilution of 1:100 were added to the plates at room temperature for 2 h. The binding reactions of antibodies against DIO2 peptides were detected with horseradish peroxidase conjugated with goat antihuman IgG in dilution of 1:5000 (SIGMA, USA) at room temperature for 1 h. Chromogenic o-phenylenediamine (OPD) substrate (SIGMA, USA) was applied for the detection in 0.05 M citrate buffer (pH 5), and the reaction was stopped with 50 µl/well of 4 N sulfuric acid after 30 min. The optical densities (O.D.) of the wells were measured with ELISA Reader at 492 nm. All tests were performed in duplicates. O.D. values above the control mean O.D. + 2 SD were regarded as positivity: 106.37 for hom-peptide antibodies and 106.67 for cys-peptide antibodies. The intra- and interassay coefficients were as follows: 4.11% and 3.54% for hom-peptide antibodies and 5.9% and 3.8% for cys-peptide antibodies, respectively.

Measurement of thyroid hormone and antibody serum levels

Thyroid hormone (TSH, FT_4, FT_3) and antibody (against TPO, Tg) serum levels were measured with chemiluminescence immunoassay in a fully automated method. The normal ranges of values were the following: 0.3–3 mIU/l for TSH, 0.7–1.48 ngl/dl for FT_4 and 1.45–3.48 pg/ml for FT_3, and 0–63 IU/ml for anti-TPO and 0–115 IU/ml for anti-Tg antibodies. Autoantibodies against TSH receptor were measured with competitive enzyme immunoassay using labeled

TSH (Medizym T.R.A., Medipan, Germany) in ELISA. The values above 1.5 IU/l were regarded as positivity.

Statistics

The descriptive results of measured thyroid hormone and antibody serum levels were exhibited in arithmetic mean ± SD. Data of age and duration of thyroid disease and ophthalmopathy or CAS were exhibited in median and interquartile range (IQR). The significance for categorical data between two or among more patient groups was assessed with Chi-squared test. The data for FT_3, FT_4 and antibodies against DIO2 peptides, TPO, Tg and TSH receptor were skewed, such that their logarithms were approximately normally distributed. Therefore, the mean is expressed as geometric mean (GM) and the error bars of GM + SD and GM − SD showing the log-transformed data mean ± SD are transformed to the original scale. Similarly, the sample's 95% confidence interval is retransformed from the mean ± 2SD of the logarithm of the data. Student paired *t*-test was applied to compare the logarithmically transformed serum thyroid hormone and antibody levels between the two groups. Regression analysis with curve estimation and 95% confidence interval of the correlation coefficient (*r*) was applied to reveal a relationship between data. *P* values below 0.05 were regarded as significant, but near significant results were also exhibited. The statistical analysis was performed with SPSS 15.0 and Medcalc 17.9.7 softwares for Windows®.

Results

Serum thyroid hormone and antibody levels in the studied patients with Graves' disease

The descriptive results in serum thyroid hormone and antibody levels were as follows in all patients with Graves' disease according to the presence and absence of ophthalmopathy at the time of the study: 0.38 ± 0.6 mIU/l for TSH, 1.67 ± 0.66 ng/dl for FT_4, 4.54 ± 2.43 pg/ml for FT_3, 181.69 ± 232.34 IU/ml for anti-TPO and 262.31 ± 695.33 IU/ml for anti-Tg antibodies, as well as 10.2 ± 13.65 IU/l for TSH receptor antibodies in patients with ophthalmopathy; 0.84 ± 3.16 mIU/l for TSH, 1.77 ± 1.28 ng/dl for FT_4, 6.35 ± 6.43 pg/ml for FT_3, 357.95 ± 408.58 IU/ml for anti-TPO and 298.03 ± 433.48 IU/ml for anti-Tg antibodies, as well as 15.3 ± 23.05 IU/l for TSH receptor antibodies in patients without ophthalmopathy. O.D. values of DIO2 peptide antibodies were as follows: 97.35 ± 4.51 for hom- and 97.05 ± 4.81 for cys-peptide antibodies.

The frequency of antibodies against DIO2 peptides and their relationships with serum thyroid hormone and antibody levels in hyperthyroidism

Cys-peptide antibodies were found in 18 cases out of 39 without and 15 cases out of 39 with ophthalmopathy, and 1 case out of 30 controls (Table 1). Hom-peptide antibodies were found in 6 cases out of 39 without and 7 cases out of 39 with ophthalmopathy, and 1 case out of 30 controls. The prevalence of anti-peptide antibodies was as follows: not found in 41 cases (52.6%), only hom-peptide antibodies in 4 cases (5.1%), only cys-peptide antibodies in 24 cases (30.8%) and antibodies directing to both peptides in 9 cases (11.5%) out of all patients with Graves' disease. Antibodies with the properties against both cys- and hom-peptides were detected in 6 patients with ophthalmopathy. The highest frequency of studied DIO2 peptide antibodies occurred in hyperthyroidism compared with those in euthyroidism (20 cases vs 11 cases for cys-peptide and 11 cases vs 2 cases for hom-peptide antibodies, of that the same antibody directed to cys- and hom-peptides in 8 cases with hyperthyroidism and 1 case with euthyroidism). In euthyroidism, the frequency of DIO2 peptide antibodies was remarkably smaller for hom-peptide antibodies than those found for cys-peptide antibodies, 1 case versus 10 cases, $P < 0.006$. In all Graves' patients, the relationship between DIO2 peptide antibodies or between cys-peptide antibodies and serum FT_4 levels was calculated. O.D. values of cys-peptide antibodies were inversely correlated with serum FT_4 levels in patients with Graves' disease ($r = -0.2607$, $P < 0.021$) (Fig. 1a). A strong correlation could be demonstrated between cys- and hom-peptide antibodies in all Graves' patients ($r = 0.3948$, $P < 0.0003$) (Fig. 1b).

The presence of anti-TPO or anti-Tg antibodies was associated with a small number of patients with cys- or hom-peptide antibodies in Graves' ophthalmopathy: 3 cases out of 21 versus 11 cases (5 cases against both peptides) out of 17, $P < 0.001$ for cys-peptide and 1 case out of 21 versus 5 cases (all cases against both peptides) out of 17, $P < 0.038$ for hom-peptide antibodies between anti-TPO antibody-positive and antibody-negative patients, as well as 0 cases out of 12 versus 6 cases (5 cases against both peptides) out of 23, $P < 0.052$ for hom-peptide antibodies between anti-Tg antibody-positive and antibody-negative patients. In hyperthyroidism, the patients with cys-peptide antibody positivity showed greater FT_3/FT_4 ratios than those who had no DIO2 peptide antibodies (0.42 [95%CI 0.2–0.88] vs 0.32 [95%CI 0.17–0.59], $P < 0.021$) (Fig. 2a). Patients with antibodies directing to both DIO2 peptides demonstrated significantly reduced serum anti-TPO (29.81 IU/ml [95%CI 1.89–471.27] vs 182.67 IU/ml [95%CI 11.22–2972.76], $P < 0.003$) and anti-Tg antibody levels compared with those who had no DIO2 peptide antibodies or who had only

Table 1 Frequency of DIO2 peptide antibodies with respect to thyroid function and the treatment with antithyroid drugs

Clinical parameters	Antibodies against					*P*
	Cys-peptide		Hom-peptide		Both peptides	
	NO	YES	NO	YES	Yes	
All studied patients						
Graves' disease ($n=78$)						
NO ($n=39$)	21	15	33	3	3	
YES ($n=39$) ophthalmopathy	24	9	32	1	6	
Controls ($n=30$)	29	1	29	1	0	
Thyroid function						
Hyperthyroidism ($n=51$)	31	12	40	3	8	
Euthyroidism ($n=24$)	13	10*	22	1*	1	0.006*
Hypothyroidism ($n=3$)	1	2	3	0	0	
Hyperthyroid Graves' patients treated with antithyroid drugs						
NO ($n=27$)	16	8	22	2	3[a]	0.008[a]
MM I ($n=45$)	29	13**	40	2	3[a]	
PTU ($n=6$)	0	3**	3	0	3[a]	0.016**
Hyperthyroid Graves' patients treated with MMI						
NO ($n=12$)	6	4[b]	9	1	2	0.015[b]
YES ($n=12$) ophthalmopathy	12	0[b]	12	0	0	

Cys-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 132 and 152; Hom-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 124 and 144; DIO2: type 2 deiodinase enzyme; MMI: methimazole; PTU: propylthiouracil

*$P < 0.006$; **$P < 0.015$

[a]$P < 0.008$

[b]$P < 0.016$

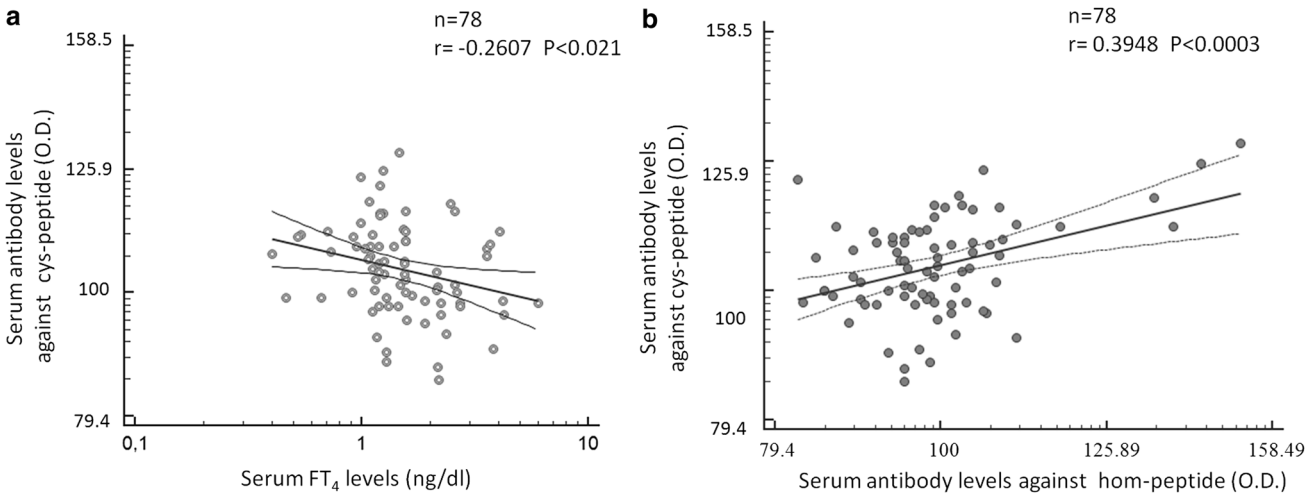

Fig. 1 Cys-peptide antibodies inversely correlated with serum FT_4 levels and positively with hom-peptide antibody levels studied in all patients with Graves' disease. Cys-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 132 and 152; hom-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 124 and 144; O.D.: optical density

cys-peptide antibodies (21.24 IU/ml [95%CI 16.26–27.44] vs 127.8 IU/ml [95%CI 6.37–2565.06], $P < 0.002$ or vs 88.49 IU/ml [95%CI 5.24–1495.43], $P < 0.013$, respectively) (Fig. 2b, c). Hyperthyroid patients with Graves' ophthalmopathy demonstrated lower serum anti-TPO (18.63 IU/ml [95%CI 12.61–27.53] vs 267.93 IU/ml [95%CI 11.41–6290.55], $P < 0.02$ for cys-peptide antibody positivity) and anti-Tg antibody levels compared with those without

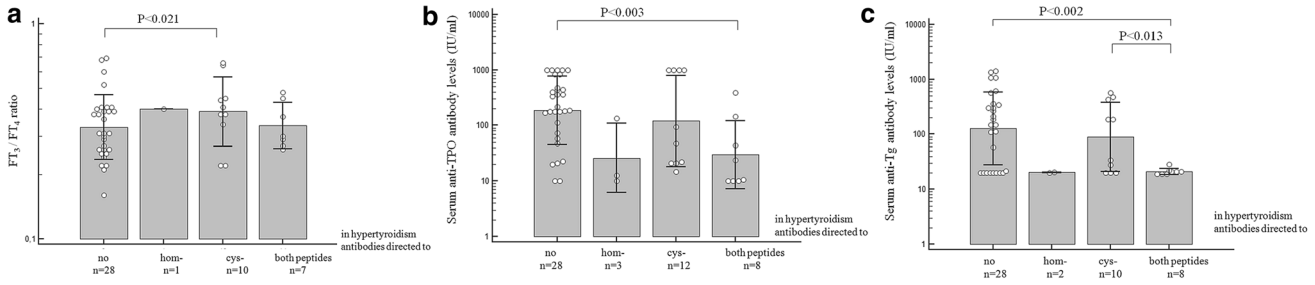

Fig. 2 Relationship between FT_3/FT_4 ratios, serum anti-TPO or anti-Tg antibody levels and DIO2 peptide antibodies in hyperthyroid Graves' patients. Cys-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 132 and 152; hom-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 124 and 144; DIO2: type 2 deiodinase enzyme; TPO: thyroid peroxidase; Tg: human thyroglobulin

ophthalmopathy (153.81 IU/ml [95%CI 8.56–2763.88] vs 238.28 IU/ml [95%CI 16.38–3468.85], $P < 0.021$ for missing DIO2 peptide antibodies) (Fig. 3a, b). The presence of hom-peptide antibodies was connected to decreased serum anti-TPO or anti-Tg antibody levels only in Graves' patients without ophthalmopathy (25.85 IU/ml [95%CI 1.55–431.99] vs 238.28 IU/ml [95%CI 16.38–3468.85], $P < 0.029$ or 20.25 IU/ml [95%CI 19.57–20.95] vs 284.82 IU/ml [95%CI 12.58–6446.01], $P < 0.045$, respectively). In ophthalmopathy, the patients with both anti-peptide antibody reactivity demonstrated remarkably decreased anti-TPO or anti-Tg levels compared with those who had no DIO2 peptide antibodies (16.21 IU/ml [95%CI 4.35–60.46] vs 153.81 IU/ml [95%CI 8.56–2763.88], $P < 0.004$ or 19.98 IU/ml [95%CI 18.11–22.04] vs 73.87 IU/ml [95%CI 6.34–860.16], $P < 0.033$, respectively), while in patients without ophthalmopathy that was found only for anti-Tg levels (22.8 IU/ml [95%CI 15.24–34.13] vs 284.82 IU/ml [95%CI 12.58–6446.01], $P < 0.021$).

The presence of TSH receptor antibodies was associated with a larger number of patients with hom-peptide antibodies in Graves' ophthalmopathy: 7 cases (6 cases against both peptides) out of 26 versus 0 cases out of 13, $P < 0.039$ between TSH receptor antibody-positive and antibody-negative patients. Surprisingly in Graves' ophthalmopathy, serum TSH receptor antibody levels decreased in patients with cys-peptide antibody positivity, but increased in patients with both cys- and hom-peptide antibody positivities compared

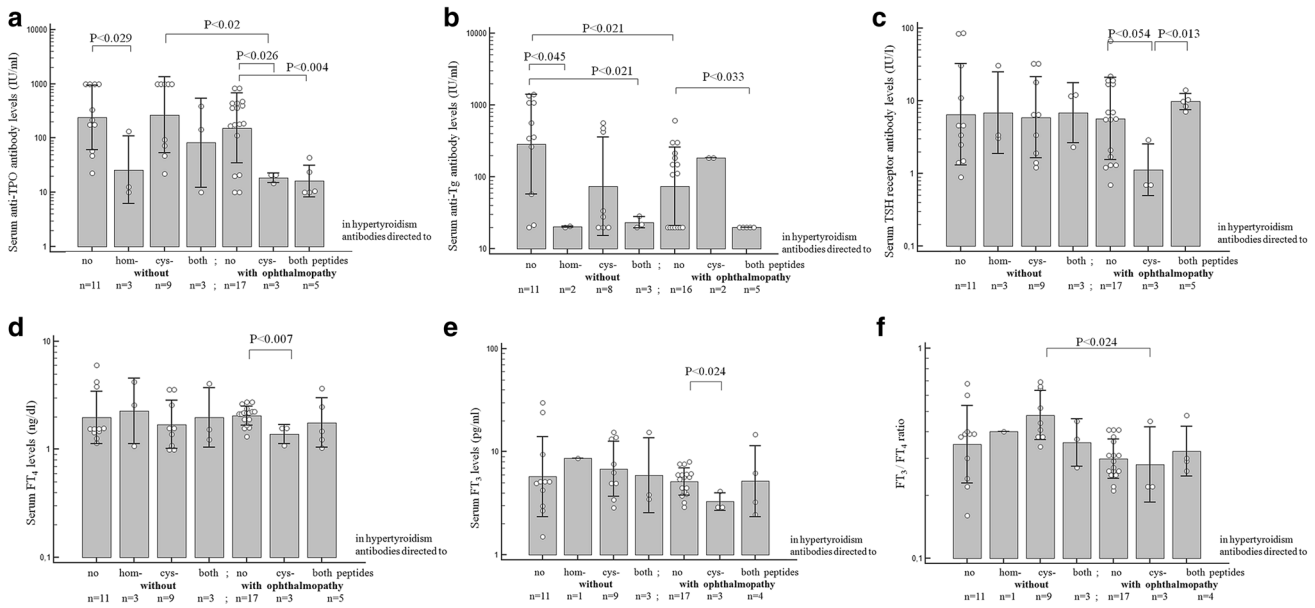

Fig. 3 Relationship between serum thyroid hormone or antibody levels and DIO2 peptide antibodies in hyperthyroid Graves' patients with respect to the presence or absence of ophthalmopathy. Cys-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 132 and 152; hom-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 124 and 144; DIO2: type 2 deiodinase enzyme; TPO: thyroid peroxidase; Tg: human thyroglobulin

with those who had no DIO2 peptide antibodies (5.68 IU/l [95%CI 0.44–72.59] vs 1.12 UI/l [95%CI 0.23–5.62], $P < 0.054$ and vs 9.73 IU/l [95%CI 5.88–16.12], $P < 0.013$). The patients with cys-peptide antibody positivity demonstrated significantly lower FT_3/FT_4 ratios in ophthalmopathy than those who had no ophthalmopathy (0.28 [95%CI 0.12–0.63] vs 0.48 [95%CI 0.28–0.37], $P < 0.024$) (Fig. 3f). In ophthalmopathy, serum FT_3 decreased rather than FT_4 levels in cys-peptide positivity compared with those who had no DIO2 peptide antibodies (3.28 pg/ml [95%CI 2.21–4.85] vs 6.83 pg/ml [95%CI 2.03–23.02], $P < 0.024$ for FT_3 and 1.38 ng/dl [95%CI 0.92–2.08] vs 2.26 ng/dl [95%CI 0.58–8.82], $P < 0.007$ for FT_4) (Fig. 3d, e).

Effect of antithyroid drugs on serum thyroid hormone and antibody levels with respect to the occurrence of antibodies against DIO2 peptides

DIO2 peptide antibodies were already present in patients with hyperthyroid Graves' disease who have not been treated yet (11 cases out of 27 against cys-peptide or 5 cases out of 27 against hom-peptide, of which 3 cases possessed antibodies against both cys- and hom-peptides). However, PTU treatment resulted in larger occurrences of DIO2 peptide antibodies compared with those given by MMI treatment [3 cases out of 6 vs 13 cases out of 45, $P < 0.016$ for cys-peptide antibodies, and 0 cases out of 6 versus 2 cases out of 45 for hom-peptide antibodies (excluding data of anti-peptide antibodies directing to both cys- and hom-peptides)]. The frequency of antibodies against both peptides was significant with respect to treatment: 3 out of 27 no treated, 3 out of 45 treated with MMI and 3 out of 6 treated with PTU, $P < 0.008$. The presence of cys-peptide antibodies was significantly smaller in patients with Graves' ophthalmopathy treated with MMI than those found in patients without eye symptoms (0 cases out of 12 vs 4 cases out of 12, $P < 0.015$), and 2 cases possessed antibodies against both cys- and hom-peptides. The presence of hom-peptide antibodies was also smaller in ophthalmopathy (0 cases out of 12). In hyperthyroidism, the presence of hom-peptide antibodies was associated with elevated serum FT4 levels compared with those who did not have DIO2 peptide antibodies, while the presence of cys-peptide antibodies was connected to reduced serum TSH receptor antibody levels compared with those who possessed antibodies against both DIO2 peptides (3.29 ng/dl [95%CI 1.64–6.57] vs 1.78 ng/dl [95%CI 1.01–3.13], $P < 0.01$ for FT_4 and 2.47 IU/l [95%CI 0.41–14.77] vs 9,99 IU/l [95%CI 5.59–17.86], $P < 0.04$ for TSH receptor antibody) (Fig. 4a, b). The cys-peptide antibody-positive patients demonstrated a relevant increase in FT_3/FT_4 ratios and serum TSH receptor antibody levels when they were treated with PTU compared with those treated with MMI (1.65 [95%CI 0.62–0.68] vs 0.4 [95%CI 0.18–1.01], $P < 0.044$ for FT_3/FT_4 ratio and 32.5 UI/l [95%CI 32.5–32.5] vs 2.68 IU/l [95%CI 0.17–41.89], $P < 0.009$ for TSH receptor antibody) (Fig. 4c, d). However, in FT_4 hyperthyroidism, MMI-treated patients with Graves' ophthalmopathy demonstrated more reduced serum FT_3 levels and FT_3/FT_4 ratios in comparison with patients who had no ophthalmopathy (4.84 pg/ml [95%CI 2.51–9.44] vs 8.14 pg/ml [95%CI 2.15–30.89], $P < 0.028$ for FT_3 and 0.31 [95%CI 0.19–0.49] vs 0.42 [95%CI 0.25–0.7], $P < 0.007$ for FT_3/FT_4 ratio) (Fig. 4d, e).

The relationship between serum thyroid hormone or antibody levels, as well as the clinical activity score (CAS), and the occurrence of antibodies against DIO2 peptides

The frequency of DIO2 peptide antibodies was investigated with the clinical signs, such as CAS and NOSPECS classifications, and the duration of thyroid disease and ophthalmopathy at the time of the study, as well as genders (Table 2). The frequency of antibodies directing to cys-, hom- or both peptides was evaluated with Chi-squared test. In the duration of ophthalmopathy, a relevant significance was found, $P < 0.044$ with the increasing patient numbers with antibodies against both peptides. In the patients with Graves' ophthalmopathy and antibodies against both peptides, the O.D. values of hom-peptide antibodies are correlated with CAS ($P < 0.033$, $r = 0.849$). In ophthalmopathy, the patients with CAS > 4 demonstrated a relevant increased serum anti-TPO antibody levels in the presence of missing DIO2 peptide antibodies compared with those who had CAS ≤ 4 (369.74 IU/ml [95%CI 124–1102.53] vs 30.8 IU/ml [95%CI 2.72–349.05], $P < 0.011$. The difference in FT_3/FT_4 ratios was not significant: 0.28 [95%CI 0.21–0.37] vs 0.34 [95%CI 0.2–0.58], $P < 0.054$ for FT3/FT4 ratio) (Fig. 5a, b). But in the presence of antibodies against both DIO2 peptides, the serum anti-TPO antibody levels decreased relevantly compared with those who had no DIO2 peptide antibodies (10.2 IU/ml [95%CI 9.55–10.89] vs 369.75 IU/ml [95%CI 124–1102.53], $P < 0.0001$) (Fig. 5d).

Discussion

Autoantibodies against DIO2 peptides can appear besides autoantibodies against TSH receptor, TPO and/or Tg in hyperthyroid Graves' disease. It seems the above-mentioned autoantibodies are associated with thyroid hormone synthesis. Nevertheless, hyperthyroidism via sympathicotonia drives the T helper (Th) balance into Th2 dominance enhancing the production of various autoantibodies too [12]. This study confirmed that DIO2 enzyme (supposedly DIO1 too) can be involved as antigen in the thyroid

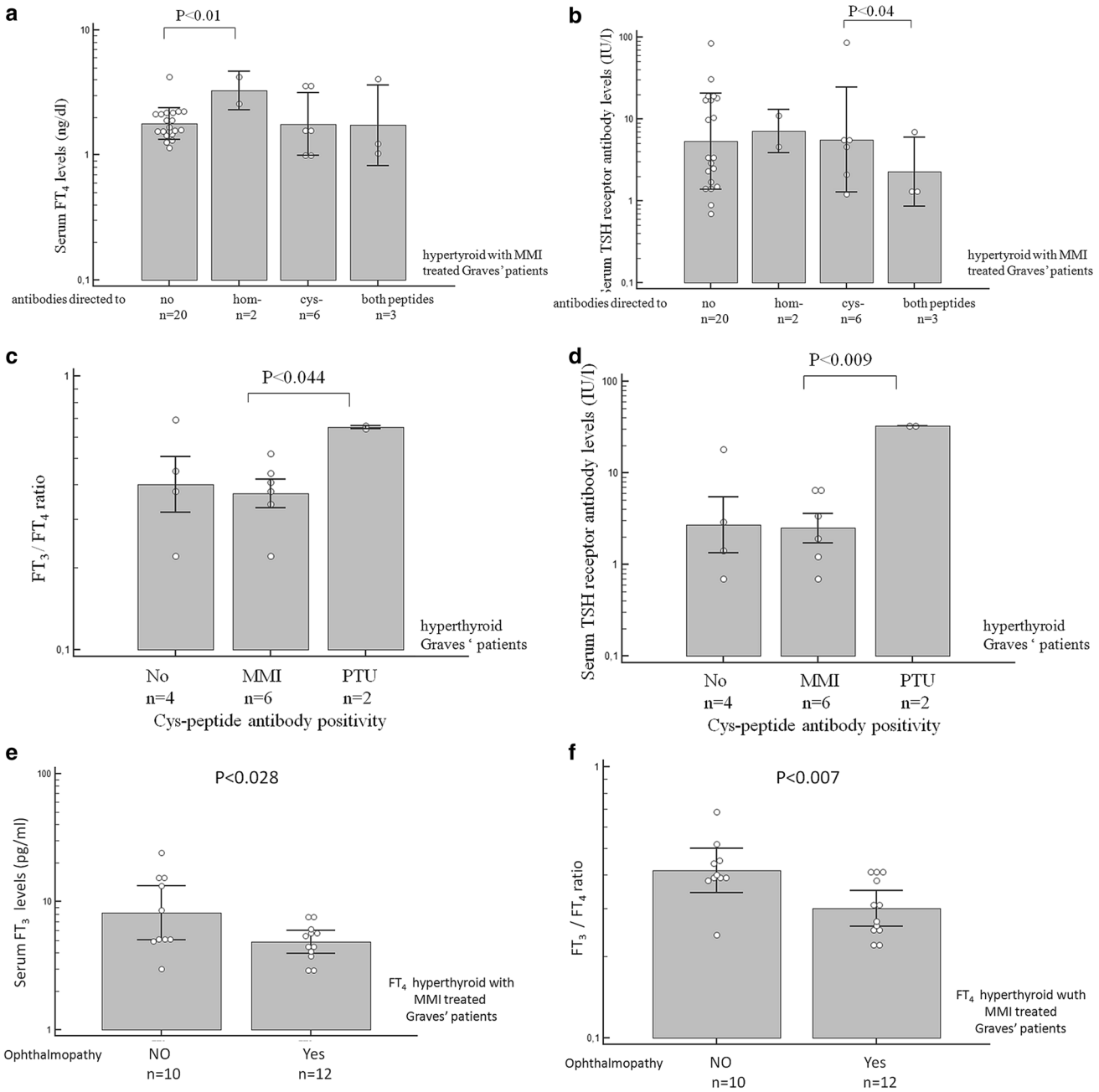

Fig. 4 Relationship among methimazole (MMI) and propylthiouracil (PTU) treatments, and thyroid hormone and TSH receptor antibody levels, as well as DIO2 peptide antibody levels in hyperthyroid Graves' disease. Cys-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 132 and 152; hom-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 124 and 144; DIO2: type 2 deiodinase enzyme

autoimmunity of Graves' disease. Antithyroid drugs block the thyroid hormone synthesis via inhibiting the activity of TPO and DIO1 enzymes leading to impairment of H_2O_2 generation and the coupling of iodotyrosines, as well as giving intermedier selenyl-iodide-DIO1 enzyme complex [13]. Both antithyroid drugs are thioamide derivates with the possibility for binding to DIO1 (supposedly to DIO2 too) and TPO enzymes modifying the enzyme actions in thyroid hormone synthesis [14, 15]. PTU is a potent DIO1 inhibitor, but MMI is a selective DIO1 blocker and also inhibits thyroid H_2O_2 generation, but they have no significant effects on DIO2 activity [16–18]. Our results demonstrated that antibodies against DIO2 cys- and hom-peptides, as well as both peptides, can develop in hyperthyroid Graves' disease and their occurrences were fewer in euthyroidism. The presence of antibodies against both peptides played a crucial role in

Table 2 Number of patients with antibodies directed to cys-, hom- and both DIO2 peptides in Graves' disease with respect to clinical activity score (CAS), NOSPECS classification, duration of thyroid disease and ophthalmopathy, as well as genders

Number of patients																			
Antibodies directed to	CAS		NOSPECS classes				Duration of thyroid disease (months)							Duration of ophthalmopathy* (months)					Genders	
	≤4	>4	2	3	23	234	0	1	2	3	4	5	6	0	1	2	3	4	Male	Female
No	8	15	1	0	12	10	5	11	12	9	3	1	2	2	7	8	4	2	12	32
Cys-peptide	0	1	0	0	0	1	1	0	3	0	0	0	0	0	0	1	0	0	0	4
Hom-peptide	6	3	0	1	5	3	3	8	5	3	2	0	0	1	2	0	0	1	4	17
Both peptides	2	4	0	0	2	4	3	3	3	0	0	0	0	2	2	2	0	0	4	5

Cys-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 132 and 152; hom-peptide: corresponding to type 2 deiodinase enzyme, aa sequences between 124 and 144; DIO2: type 2 deiodinase enzyme; NOSPECS class 2: soft tissue involvement; class 3: proptosis; class 4: eye muscle enlargements; duration of thyroid disease or ophthalmopathy: 1: 1–5 months; 2: 6–10 months; 3:11–15 months; 4: 16–20 months; 5: 21–25 months; 6: 26–30 months

*$P < 0.044$ by Chi-squared test

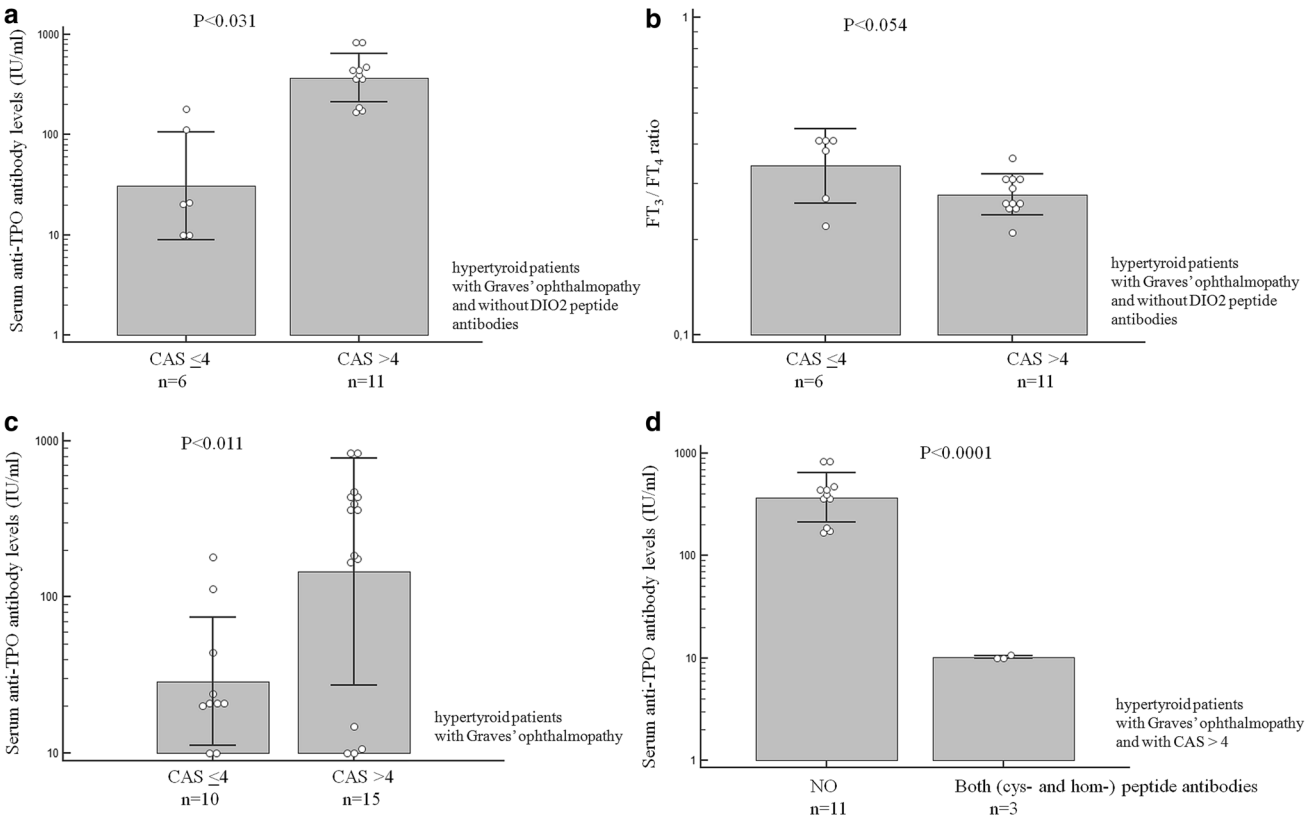

Fig. 5 Relationship between the clinical activity score (CAS) for ophthalmopathy, the serum anti-TPO levels or FT_3/FT_4 ratios and DIO2 peptide antibodies. DIO2: type 2 deiodinase enzyme; TPO: thyroid peroxidase

the reduced serum anti-TPO, anti-Tg and TSH receptor antibody levels, as well as their occurrences mainly connected to hyperthyroidism, particularly to ophthalmopathy. Hyperthyroid Graves' patients treated with PTU demonstrated larger frequency of antibodies against DIO2 peptides, suggesting that the blocking effect of PTU on DIO1, but not on DIO2 can promote the development of DIO2 peptide antibodies [4]. Our results demonstrated a distinct effect of antithyroid drugs on the presence and degree of TSH receptor antibody levels highlighting the complementary role of DIO2 antibodies in the development of hyperthyroidism. MMI-treated hyperthyroid Graves' patients demonstrated reduction in the frequency of antibodies against DIO2 peptides, particularly in Graves' ophthalmopathy, which may be connected to the

fact that only DIO2 was expressed in extraocular muscles [19]. The appearance of DIO2 peptide antibodies was also remarkably smaller in Graves' ophthalmopathy, but they possessed larger patient numbers with antibodies directing to both peptides confirming the dominant role of DIO1 enzyme in Graves' hyperthyroidism and the difference in the binding of thioamide to deiodinase reactions. The presence of DIO2 peptide antibodies is increased by PTU and decreased by MMI treatment in Graves' hyperthyroidism, demonstrating that their productions are connected to thyroid hormone synthesis. Not only the treatment with antithyroid drugs highlights the relationship between Graves' hyperthyroidism and the occurrence of antibodies against DIO2 peptides, but also their simultaneous occurrences with anti-TPO, anti-Tg or TSH receptor antibodies. In Graves' ophthalmopathy, our data showed the presence of antibodies against DIO2 peptides was associated with smaller frequency of anti-TPO and anti-Tg antibodies, but greater frequency of TSH receptor antibodies. These data are in concordance with the clinical findings. Decreased anti-TPO and increased TSH receptor antibody levels could be found in patients with Graves' ophthalmopathy compared with those without ophthalmopathy [20, 21]. The difference in the occurrence of DIO2 peptide antibodies between Graves' patients with and without ophthalmopathy may be influenced by cytokines, such as IL-1, IL-6 and IFNγ playing an inhibitory role in DIO1 and DIO2 enzyme activities [22]. Our previous study demonstrated that hyperthyroid Graves' patients with ophthalmopathy possessed greater serum IL-6 levels than those who did not have ophthalmopathy [23]. The appearance of DIO2 antibodies can influence the serum thyroid hormone and antibody levels of patients. An inverse correlation was found between serum FT_4 levels and O.D. values of cys-peptide antibodies in Graves' disease, while the presence of hom-peptide antibodies was connected to increased serum FT_4 levels, highlighting that DIO2 peptide antibodies may have inhibiting or stimulating properties. Surprisingly, the missing DIO2 peptide antibodies in hyperthyroid Graves' ophthalmopathy demonstrated that the MMI treatment was connected to significantly lower serum FT_3 levels and FT_3/FT_4 ratios leading to faster fall in thyroid hormone levels. CAS above 4 was associated with higher serum levels of anti-TPO antibodies, which were remarkably decreased in the presence of antibodies against both peptides highlighting the importance of DIO2 peptide antibodies in ophthalmopathy.

Nakahara and coworkers investigated the presence of DIO2 peptide (aa sequences were the same to hom-peptide in 100%) and antipituitary (APA) antibodies in patients with Hashimoto's thyroiditis and Graves' disease [24]. In Graves' disease, they found that 9 cases out of 68 (13.2%) showed antibodies against both APA and DIO2 peptides, while 18 cases out of 68 patients with Graves' disease (26.5%) and 11 cases out of 42 patients with Hashimoto's thyroiditis (26.2%) demonstrated DIO2 peptide antibodies, independently of thyroid function.

DIO2 polymorphism may influence the development of antibodies against DIO2 peptides. The well-known single-nucleotide polymorphisms: Thr92ALA (rs225014), ORFa-Gly3Asp (rs12885300) and others (rs225012, rs225010) do not affect the studied DIO2 aa sequences [25–27]. However, Bianco and coworkers confirmed that the selenium at position 133 (Sec133) plays a crucial structural role: the cys for Sec substitution at position 133 of human DIO2 increased 500- to 1000-fold its enzymatic activity, but Sec133Ala exchange inactivates the enzyme activity [28]. Our DIO2 peptides were substituted by cys (hom-peptide) or cyscys (cys-peptide) amino acids at position 133.

The different effect of antithyroid drugs on deiodination was investigated by Abuid and Larsen, where they demonstrated that PTU treatment led to greater inhibition of thyroid hormone synthesis via blocking the peripheral T_3 production too [29]. However, the inhibition of DIO1 enzyme by PTU is competitive and influenced by its dithiothreitol (DTT) cosubstrate or other reductive cofactors, such as cobalamin, flavoenzyme–NADPH system, dehalogenation mechanisms, selenium or sulfur halide [30]. The study using Se- and S-based PTU and MMI analogs suggested multiple pathways in the inhibition of DIO2 activity [31].

Conclusions

Autoantibodies against DIO2 peptides are present in hyperthyroid Graves' disease besides autoantibodies against TSH receptor, TPO and/or Tg. It seems the enzymes (TPO, DIO2), which are implicated in thyroid hormone synthesis, can be a source of autoantigens in the autoimmunity of Graves' disease. The occurrence of DIO2 peptide antibodies is differently influenced by PTU and MMI treatments, as well as by the thyroid function. In Graves' ophthalmopathy, the presence of DIO2 peptide antibodies was connected to smaller frequency or lower serum levels of anti-TPO antibodies, as well as to greater frequency and serum levels of TSH receptor antibodies. The data highlighted that cys-peptide antibodies may have inhibiting, but hom-peptide antibodies stimulating properties, and antibodies against both peptides are inhibiting for anti-TPO and anti-Tg antibodies. PTU treatment led to greater frequency of DIO2 peptide antibodies and the degree of TSH receptor antibodies than those found by MMI treatment, particularly in Graves' ophthalmopathy. DIO2 peptide antibodies can also influence the therapeutic efficacy in hyperthyroid Graves' disease.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standard of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study, formal consent was not required.

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