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Frequent Appearance of Autoantibodies Against Prohormone Convertase 1/3 and Neuroendocrine Protein 7B2 in Patients with Nonfunctioning Pituitary Macroadenoma

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Among pituitary disorders having mass effect of the pituitary gland, nonfunctioning pituitary macroadenoma and lymphocytic hypophysitis are difficult to differentiate without histological examination. In order to efficiently distinguish lymphocytic hypophysitis and pituitary tumors, we studied the presence of autoantibodies against prohormone-processing enzymes, prohormone convertase (PC) 1/3, PC2, carboxypeptidase E (CPE), and PC2 regulatory protein, 7B2, by radioligand assay using recombinant human ^{35}S-labeled protein in patients with clinically nonfunctioning pituitary macroadenoma, lymphocytic hypophysitis, and other pituitary diseases. The indexes for anti-PC1/3 antibodies (Ab) were significantly higher in patients with nonfunctioning pituitary macroadenoma than in patients with lymphocytic hypophysitis. Patients positive for either anti-PC1/3 or anti-7B2 Ab were significantly frequent among patients with nonfunctioning pituitary macroadenoma than in other pituitary diseases and healthy controls. None of the patients was positive for anti-PC2 Ab or anti-CPE Ab. These results suggest that autoantibodies against PC1/3 and 7B2 are novel tumor-associated autoantibodies and can be helpful in the diagnosis of clinically nonfunctioning pituitary macroadenoma.

Key Words: Antipituitary autoantibodies; nonfunctioning pituitary macroadenoma; lymphocytic hypophysitis; tumor-associated autoantibodies.

Introduction

In pituitary disorders with mass effect on the pituitary gland, nonfunctioning pituitary macroadenoma and lymphocytic hypophysitis are difficult to differentiate, and histological examination is often required for a definite diagnosis. In order to distinguish pituitary tumors from lymphocytic hypophysitis, the use of specific biochemical or serological markers is gaining increased attention. Autoantibodies are candidates for such specific serological markers.

On one hand, autoantibodies are useful for the diagnosis of various autoimmune diseases. In pituitary disorders, antipituitary autoantibodies have been surveyed to help diagnose patients with lymphocytic hypophysitis. Antipituitary autoantibodies were originally detected by a complement fixation test in a study of Sheehan's syndrome *(1)*. The immunoblotting method using fractions of pituitary specimens helped to identify the components of autoantigens, and identified autoantigens of a 22 kDa protein and a 49 kDa protein as growth hormone (GH) and alpha-enolase, respectively, in patients with autoimmune hypophysitis and related diseases *(2–5)*.

On the other hand, autoantibodies associated with tumors have been reported after the first report of p53 *(6,7)*. In pituitary tumors, smooth muscle Ab (SMA) *(8)*, anti-ACTH and anti-TSH Ab *(9)*, 68-kDa autoantigen *(10)*, and 49-kDa pituitary cytosolic protein *(4)* have been reported.

Recently, we identified two novel pituitary-specific proteins, pituitary gland specific factor (PGSF) 1a and PGSF2 *(11)*, and detected autoantibodies against them in patients with lymphocytic hypophysitis and other hypopituitarism using radioligand assay (RLA) *(12)*. Autoantibodies are often produced against enzymes such as glutamic acid decarboxylase (GAD) and islet cell antigen-2 (IA-2) for type I diabetes and thyroid peroxidase (TPO) for Hashimoto's thyroiditis. As anti-alpha-enolase Ab was detected in patients with autoimmune hypophysitis *(5)*, we recently studied anti-alpha-enolase Ab by RLA and detected them both in patients with lymphocytic hypophysitis and those with nonfunction-

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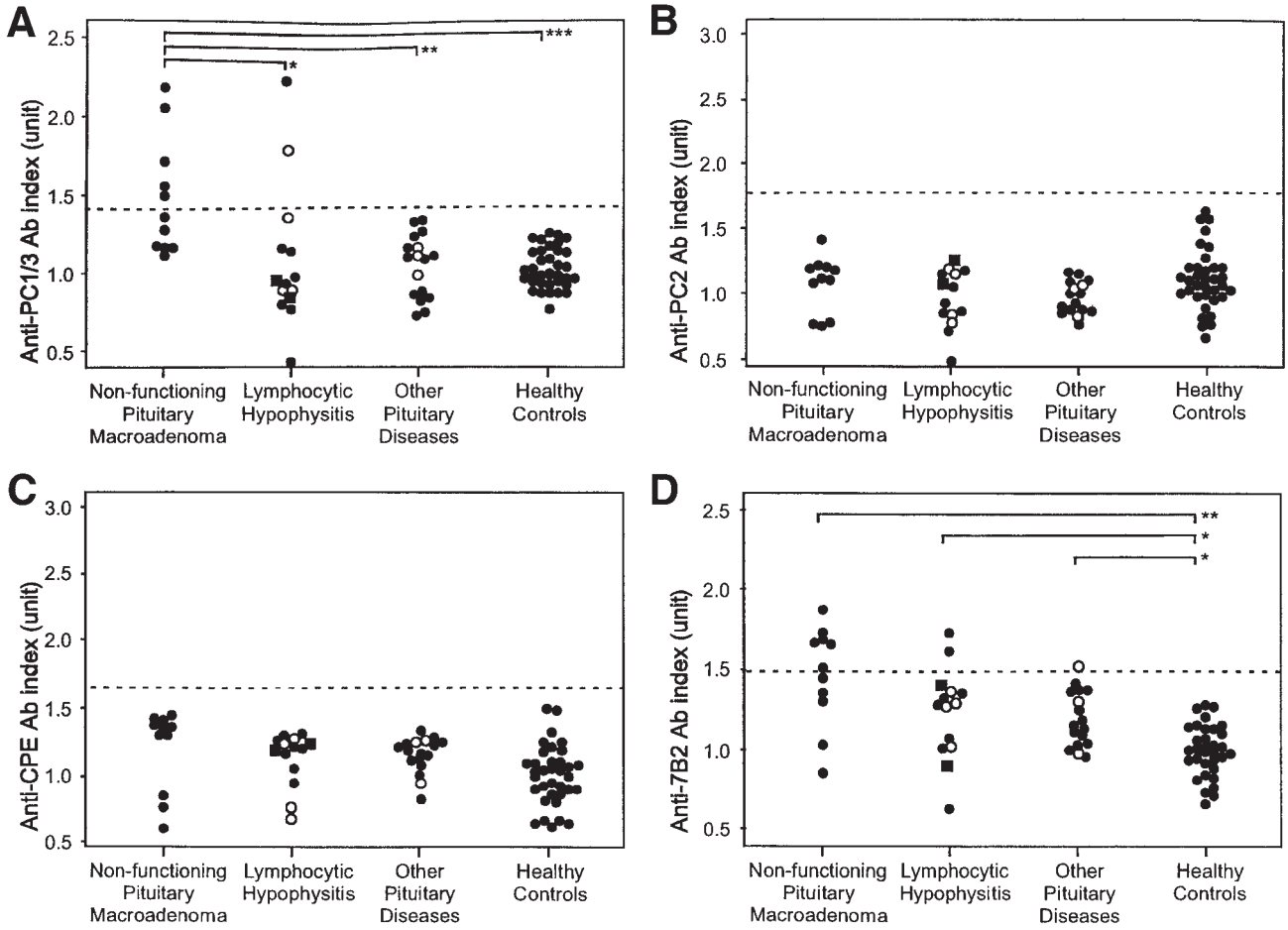

Fig. 1. Anti-PC1/3, PC2, CPE, and 7B2 Ab indexes in sera obtained from patients with pituitary disorders. (**A**) Anti-PC1/3 Ab indexes are from sera of patients with nonfunctioning pituitary macroadenoma (n = 11), lymphocytic hypophysitis (n = 14), other pituitary diseases (n = 17), and sera obtained from healthy subjects (n = 36). Open circles indicate indexes from sera of patients with lymphocytic adenohypophysitis or Sheehan's syndrome. Closed boxes indicate indexes from sera of patients with lymphocytic infundibuloneurohypophysitis proven by biopsy. The horizontal dotted line shows the cut-off value of 1.41 units. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$. (**B**) Anti-PC2 Ab indexes are shown as for the above mentioned patients. The horizontal dotted line shows the cut-off value of 1.82 units. (**C**) Anti-CPE Ab indexes are shown as for the above mentioned patients. The horizontal dotted line shows the cut-off value of 1.67 units. (**D**) Anti-7B2 Ab indexes are shown as for the above mentioned patients. The horizontal dotted line shows the cut-off value of 1.49 units.

ing pituitary macroadenoma *(13)*. To further study autoantibodies as candidate serological markers that may be used to efficiently diagnose various pituitary disorders, we further measured autoantibodies against prohormone-processing enzymes that are commonly produced in the pituitary gland, PC1/3, PC2, CPE, and PC2 regulatory protein, 7B2, in patients with nonfunctioning pituitary macroadenoma, lymphocytic hypophysitis, and other pituitary diseases, and compared them with autoantibodies against GH, PGSF1a, PGSF2, and alpha-enolase.

Results

Antipituitary Autoantibodies in Pituitary Disorders

We examined serum samples obtained from patients with pituitary disorders and compared the results with those of healthy controls (Fig. 1). The indexes for anti-PC1/3 Ab were statistically significantly higher in patients with nonfunctioning pituitary macroadenoma than in patients with lymphocytic hypophysitis and other pituitary diseases and in healthy controls, and those for anti-7B2 Ab were statistically significantly higher in patients with nonfunctioning pituitary macroadenoma, lymphocytic hypophysitis, and other pituitary diseases than in healthy controls.

With cut-off values calculated as the mean + three SD of healthy controls (anti-PC1/3 Ab 1.41 units; anti-PC2 Ab 1.82 units; anti-CPE Ab 1.67 units; anti-7B2 Ab 1.49 units), none of the serum from healthy controls tested positive (Fig. 1). Anti-PC1/3 Ab was found in 45% (5/11) and 14% (2/14) of patients with nonfunctioning pituitary macroadenoma and lymphocytic hypophysitis, respectively. Anti-7B2 Ab was found in 55% (6/11), 14% (2/14), and 33% (1/3) of

Table 1
Patients Positive for Anti-PC1/3 Ab

Diagnosis			Autoantibody					Deficient hormones and others
	Age	Sex	PC1/3	hGH	PGSF1a	PGSF2	alpha-enolase	
Nonfunctioning pituitary macroadenoma								
	66[a]	female	1.50	–	–	–	+	GH
	37	female	2.20	–	–	–	+	Normal
	54	female	2.07	–	–	–	+	Normal
	36	female	1.72	–	–	–	+	Normal
	48	female	1.57	–	–	–	–	Normal
Lymphocytic adenohypophysitis								
	77	female	1.80	–	–	–	–	Mild hyperprolactinemia[b]
Lymphocytic infundibuloneurohypophysitis								
	56	male	2.25	–	–	–	–	AVP

AVP; vasopressin.
[a]Positive for both anti-PC1/3 Ab and anti-7B2 Ab.
[b]She was diagnosed with lymphocytic adenohypophysitis as she had mild hyperprolactinemia with gadolinium-enhanced pituitary and stalk swelling *(19)*.

Table 2
Patients Positive for Anti-7B2 Ab

Diagnosis			Autoantibody					Deficient hormones and others
	Age	Sex	7B2	hGH	PGSF1a	PGSF2	alpha-enolase	
Nonfunctioning pituitary macroadenoma								
	66[a]	female	1.65	–	–	–	+	GH
	59	female	1.87	–	–	–	–	Normal
	75	male	1.72	–	–	–	–	LH
	61	female	1.68	–	–	–	–	GH, LH
	45	female	1.67	–	–	–	–	Normal
	54	female	1.51	–	–	–	–	Normal
Lymphocytic infundibuloneurohypophysitis								
	28	male	1.61	–	–	+	–	LH, FSH, ACTH, AVP, ANF(+), anti-DNA Ab(+)
	14	male	1.73	–	–	–	–	FSH, AVP
Sheehan's syndrome								
	69	female	1.52	–	–	–	+	ACTH, TSH, GH, LH, FSH, PRL, postpartum onset

ANF; anti-nucleic factor.
[a]Positive for both anti-PC1/3 Ab and anti-7B2 Ab.

patients with nonfunctioning pituitary macroadenoma, lymphocytic hypophysitis, and Sheehan's syndrome, respectively. None of the patients was positive for anti-PC2 Ab and anti-CPE Ab. The frequencies of positive reactions to either anti-PC1/3 or anti-7B2 Ab were statistically significantly higher in patients with nonfunctioning pituitary macroadenoma than in patients with other pituitary diseases and in healthy controls. When the frequencies of positive reactions to either anti-PC1/3 or anti-7B2 Ab were combined, they were also statistically significantly higher in patients with nonfunctioning pituitary macroadenoma than in patients with lymphocytic hypophysitis, in addition to patients with other pituitary diseases and in healthy controls.

Data from all patients positive for anti-PC1/3 or anti-7B2 Ab are summarized in Tables 1 and 2.

Discussion

In order to help diagnose lymphocytic hypophysitis from pituitary tumors without aggressive surgery and in order to understand the prevalence of autoantibodies in various pituitary disorders, numerous studies have sought to identify antipituitary autoantibodies that could be used as noninvasive serological markers. Recently, we identified two novel pituitary-specific antigens, PGSF1a and PGSF2 *(11)*, and detected autoantibodies against these proteins in patients

with lymphocytic hypophysitis and other hypopituitarism, but not in patients with nonfunctioning pituitary macroadenoma *(12)*. In this study, as autoantibodies are often produced against enzymes, we further measured autoantibodies against hormone-processing enzymes, PC1/3, PC2, CPE, and 7B2, in patients with nonfunctioning pituitary macroadenoma, lymphocytic hypophysitis, and other pituitary diseases.

In contrast to anti-PGSF1a Ab and anti-PGSF2 Ab that were detected in patients with lymphocytic hypophysitis but not in patients with nonfunctioning pituitary macroadenoma *(12)*, and anti-alpha-enolase Ab that was detected in both patients with lymphocytic hypophysitis and those with nonfunctioning pituitary macroadenoma *(13)*, autoantibodies against PC1/3 and 7B2 were predominantly detected in patients with nonfunctioning pituitary macroadenoma, while autoantibodies against PC2 and CPE were not detected in any of the pituitary disorders (Fig. 1). As the serum samples were obtained directly after pituitary surgeries, autoantibodies observed in these patients are not due to surgical neoexposure of antigens. Thus, the production of autoantibodies varies between tumor and autoimmune diseases, probably due to the difference in recognition or presentation of antigens between lymphocytic hypophysitis and nonfunctioning pituitary macroadenoma.

Although lymphocytic hypophysitis can only be proved histologically, when patients were suspected of having lymphocytic adenohypophysitis with consistent clinical findings and MRI features and without visual field defects, therapeutic trials with steroid would be justified without biopsy *(14–16)*. In patients with biopsy-proven lymphocytic adenohypophysitis, intensely enhancing pituitary masses in MRI have been reported in most cases *(14,17,18)*, and so we used it as the diagnostic criteria for suspected lymphocytic adenohypophysitis. According to this diagnostic criteria, autoantibodies were detected similarly both in patients with biopsy-proven and clinically suspected lymphocytic hypophysitis *(19)*. The absence of both anti-PC1/3 and anti-7B2 Ab in the two biopsy-positive patients in this report would be another supplementary finding that our criteria are adequate for the present study.

In respect to pituitary disease, most autoantibodies so far identified have been focused on diagnosing lymphocytic hypophysitis or Sheehan's syndrome *(1–5,12)*. In this study, the indexes for anti-PC1/3 Ab were statistically significantly higher in patients with nonfunctioning pituitary macroadenoma than in patients with lymphocytic hypophysitis.

When focusing on cases positive for autoantibodies against hormone-processing enzymes, 10 out of 11 patients with nonfunctioning pituitary macroadenoma were positive for autoantibodies against either PC1/3 or 7B2, when combined, in contrast to 4 out of 14 patients with lymphocytic hypophysitis (Tables 1 and 2). Other pituitary autoantibodies against GH, PGSF1a, and PGSF2 often appeared concurrently in our previously study *(12)*, but was observed concurrently in only one patient positive for autoantibodies against PC1/3 and 7B2 (Tables 1 and 2). Thus, our present and previous data clearly show that nonfunctioning pituitary macroadenoma and lymphocytic hypophysitis are statistically distinguishable by serological markers PC1/3, 7B2, GH, PGSF1a, and PGSF2.

To date, tumor-associated autoantibodies have been documented for anti-p53 Ab *(7)* or those associated with paraNeoplastic neurological syndromes, such as anti-Hu Ab, anti-Yo Ab, anti-Ma/Ta Ab, anti-Ri Ab, anti-Tr Ab, anti-amphiphysin Ab, PCA-2, anti-CRMP5/CV2 Ab, and ANNA-3 *(20)*. These tumor-associated autoantibodies helped to substantiate paraneoplastic neurological syndromes *(20)*. Similarly, autoantibodies against PC1/3 and 7B2 can also possibly be helpful candidates for the diagnosis of paraneoplastic neurological syndromes.

In conclusion, autoantibodies against PC1/3 and 7B2 are tumor-associated and can be useful serological markers to distinguish nonfunctioning pituitary macroadenoma from lymphocytic hypophysitis when combined.

Material and Methods

Subjects

Serum samples were obtained from 11 patients with clinically nonfunctioning pituitary macroadenoma, 14 with lymphocytic hypophysitis, all of whom had pituitary enlargement (4 with lymphocytic adenohypophysitis and 10 with lymphocytic infundibuloneurohypophysitis, including 2 of the latter group proven by biopsy), and 17 with other pituitary diseases (10 patients with isolated ACTH deficiency, 4 patients with idiopathic TSH deficiency, and 3 patients with Sheehan's syndrome), and 36 healthy controls. Clinically nonfunctioning pituitary macroadenoma was diagnosed by histological examination, and serum samples were obtained directly after pituitary surgeries were performed. Lymphocytic adenohypophysitis was suspected by pituitary dysfunction associated with an intrasellar mass, which demonstrated gadolinium enhancement on MRI. Lymphocytic infundibuloneurohypophysitis was diagnosed by the presence of central diabetes insipidus, with swelling of the posterior pituitary or pituitary stalk on MRI. We obtained informed consent from all patients. The mean ages and sex distribution are summarized in Table 3. The mean ages of the groups of patients were not significantly different from the mean age of the healthy controls.

Detection of Autoantibodies Using Radioligand Assay

Autoantibodies against PC1/3, PC2, CPE, and 7B2 were determined by radioligand assay as previously described *(12)*. Briefly, cDNAs for each antigen were amplified from human pituitary gland cDNA by PCR using the following primer pairs containing either an *Eco*RI, *Hind*III, or a *Xho*I site: 7B2, 5'-GGAATTCATGGTCTCCAGGATGGTCTC

Table 3
Clinical Characteristics of the Patients and Healthy Controls

Subjects	Number examined (male/female)	Ages (yr) Range (Means ± SD)
Nonfunctioning pituitary macroadenoma[a]	11 (1/10)	36–75 (49.9 ± 11.8)
Lymphocytic hypophysitis with pituitary enlargement		
lymphocytic adenohypophysitis (suspected by MRI)	4 (1/3)	31–77 (56.5 ± 19.4)
lymphocytic infundibuloneurohypophysitis		
proven by biopsy	2 (1/1)	33–49 (41.0 ± 11.3)
suspected by MRI	8 (4/4)	14–56 (35.1 ± 16.5)
Other pituitary disease		
Isolated ACTH deficiency	10 (7/3)	20–81 (54.3 ± 16.9)
Idiopathic TSH deficiency	4 (1/3)	27–70 (56.3 ± 20.3)
Sheehan's syndrome	3 (0/3)	42–69 (60.0 ± 15.6)
Healthy controls	36 (18/18)	24–74 (49.9 ± 13.8)

[a]Range (means ± SD) of the size of pituitary macroadenoma was 1.0–3.0 cm (1.62 ± 0.56).

TAC and 5'-CCGCTCGAGTTACTCTGGATCCTTATC CTC; PC1/3, 5'-CCCAAGCTTGCATGGAGCGAAGAG CCTGGA and 5'-CCGCTCGAGCGCAGGGTAAGGAA GAAGCAT; PC2, 5'-GGAATTCATGAAGGGTGGTTG TGTCTCCCA and 5'-CCGCTCGAGCTAGTTCTTGTT AAGGATGCT; CPE, 5'-GGAATTCATGGCGTGCCCG TCTCTCCGCCG and 5'-CCGCTCGAGCCTAGGATGG CAAAATTACTT. (The *Eco*RI, *Hind*III, and *Xho*I sites have been underlined.) After amplification, the PCR products were digested with *Eco*RI or *Hind*III and *Xho*I, and ligated into the pET 28a (+) expression vector (Novagen, Madison, WI). Antigens were translated in vitro using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) and L-[^{35}S]-methionine (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions. The products were applied to Nick columns (Amersham Pharmacia Biotech) in order to remove free [^{35}S]-methionine and analyzed by SDS-PAGE (15% polyacrylamide gel), and bands were detected by autoradiography. The fractions containing each of the ^{35}S-labeled antigens were diluted with reaction buffer (150 m*M*/L NaCl, 50 m*M*/L Tris, pH 7.4, 1 mL/L Tween-20, 4 g/L bovine serum albumin, and 1 g/L NaN_3) and were stored at –80°C until use. The patients' sera (dilution 1:50) and reaction mixtures containing 20,000 counts per minute (cpm) of labeled antigen were incubated overnight at 4°C, in a total volume of 50 µL, in reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% BSA, 0.1% Tween-20, and 0.1% NaN_3, pH 7.4). The labeled antigen–Ab complex was transferred onto a 96-well filtration plate (Multiscreen HVPP, 0.45 µm, Millipore Corp.) and precipitated with Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech), which was blocked with blocking buffer (150 m*M*/L NaCl, 50 m*M*/L Tris, pH 7.4, 1 ml/L Tween-20, 30 g/L bovine serum albumin, and 1 g/L NaN_3). The labeled antigen–Ab–Protein G-Sepharose complex was washed 10 times with washing buffer (150 m*M*/L NaCl, 50 m*M*/L Tris, pH 7.4, 10 mL/L Tween-20) using a 96-well filtration system. The plate was then dried and OptiPhase SuperMix (PerkinElmer Life Science, Boston, MA) was added to each well. The quantity of precipitated ^{35}S-labeled antigen was counted using a 1450 MicroBeta TriLux apparatus (PerkinElmer Life Science). All samples were assayed in duplicate. Levels of antibodies were expressed as indexes, which were calculated as follows:

$$\text{Anti-antigen Ab index (unit)} = \frac{\text{cpm of the unknown serum}}{\text{cpm of the pooled serum of healthy controls}}$$

The cut-off value designating positive reactions was chosen as the mean + three SD of the 36 healthy controls.

Statistical Analysis

Indexes of subjects and of healthy controls were compared using the Mann–Whitney *U*-test. A p value of less than 0.01 was considered statistically significant. Frequencies of positive sera among patient groups and healthy controls were compared by Fisher's exact probability test. Estimated alpha less than 0.05 was considered statistically significant.

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References

1. Engelberth, O. and Jezkova, Z. (1965). *Lancet* **1,** 1075.
2. Kikuchi, T., Yabe, S., Kanda, T., and Kobayashi, I. (2000). *Endocr. J.* **47,** 407–416.
3. Takao, T., Nanamiya, W., Matsumoto, R., Asaba, K., Okabayashi, T., and Hashimoto, K. (2001). *Horm. Res.* **55,** 288–292.
4. Crock, P. A. (1998). *J. Clin. Endocrinol. Metab.* **83,** 609–618.

5. O'Dwyer, D. T., Smith, A. I., Matthew, M. L., et al. (2002). *J. Clin. Endocrinol. Metab.* **87,** 752–757.
6. Crawford, L. V., Pim, D. C., and Bulbrook, R. D. (1982). *Int. J. Cancer* **30,** 403–408.
7. Soussi, T. (2000). *Cancer Res.* **60,** 1777–1788.
8. Sheikh, K. M., Weiss, M. H., and Quismorio, F. P. (1983). *Neurol. Res.* **5,** 1–12.
9. Mau, M., Phillips, T. M., and Ratner, R. E. (1993). *Clin. Endocrinol. (Oxf.)* **38,** 495– 500.
10. Sawicka, J., Gryczynska, M., Baumann-Antczak, A., Gut, P., Polanska, A., and Kosowicz, J. (1999). *Pol. Arch. Med. Wewn.* **101,** 123–129 (in Polish).
11. Tanaka, S., Tatsumi, K., Okubo, K., et al. (2002). *J. Mol. Endocrinol.* **28,** 33–44.
12. Tanaka, S., Tatsumi, K., Kimura, M., et al. (2002). *Eur. J. Endocrinol.* **147,** 767–775.
13. Tanaka, S., Tatsumi, K., Takano, T., et al. (2003). *Endocr. J.* **50,** 697–702.
14. Ahmadi, J., Meyers, G. S., Segall, H. D., Sharma, O. P., and Hinton, D. R. (1995). *Radiology* **195,** 30–34.
15. Hashimoto, K., Takao, T., and Makino, S. (1997). *Endocr. J.* **44,** 1–10.
16. Beressi, N., Beressi, J. P., Cohen, R., and Modigliani, E. (1999). *Ann. Med. Int. (Paris)* **150,** 327–341.
17. Powrie, J. K., Powell, M., Ayers, A. B., Lowy, C., and Sonksen, P. H. (1995). *Clin. Endocrinol. (Oxf.)* **42,** 315–322.
18. Kristof, R. A., Van Roost, D., Klingmuller, D., Springer, W., and Schramm, J. (1999). *J. Neurol. Neurosurg. Psychiatry* **67,** 398–402.
19. Nishiki, M., Murakami, Y., Ozawa, Y., and Kato, Y. (2001). *Clin. Endocrinol. (Oxf.)* **54,** 327–333.
20. Voltz, R. (2002). *Lancet Neurol.* **1,** 294–305.