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ORIGINAL ARTICLE

Novel autoantigens in autoimmune hypophysitis

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Summary

Background Pituitary autoantibodies are found in autoimmune hypophysitis and other conditions. They are a marker of pituitary autoimmunity but currently have limited clinical value. The methods used for their detection lack adequate sensitivity and specificity, mainly because the pathogenic pituitary autoantigen(s) are not known and therefore antigen-based immunoassays have not been developed.

Objectives This study aimed to identify novel pituitary autoantigens using sera as probes in proteomic assays. We also compared immunoblotting and immunofluorescence methods for their accuracy in diagnosing autoimmune hypophysitis.

Study design and subjects Twenty-eight sera from autoimmune hypophysitis cases (14 histologically proven and 14 clinically suspected) were compared to 98 sera from controls, which included 14 patients with pituitary adenomas, 48 with autoimmune thyroiditis (15 Graves' disease and 33 Hashimoto's thyroiditis) and 36 healthy subjects.

Methods All sera were tested against human pituitary cytosolic proteins separated by one-dimensional (1D) gel electrophoresis. The band recognition was analysed statistically to detect molecular weight regions preferentially recognized by hypophysitis sera. 2D gel immunoblotting and mass spectrometry were then used to sequence the protein spots of interest. Sera were also tested by immunofluorescence for their recognition of *Macaca mulatta* pituitary sections.

Results A single region in the 25–27-kDa range was recognized more often by hypophysitis cases than healthy subjects ($P = 0{\cdot}004$) or patients with pituitary adenomas ($P = 0{\cdot}044$). This region contained two novel candidate autoantigens: chromosome 14 open reading frame 166 (C14orf166) and chorionic somatomammotrophin. Immunoblotting positivity for the 25–27-kDa region yielded greater sensitivity (64% *vs.* 57%) and specificity (86% *vs.* 76%) than immunofluorescence in predicting histologically proven hypophysitis, although the performance was still inadequate to make immunoblotting a clinically useful test.

Conclusion The study reports two novel proteins that could act as autoantigens in autoimmune hypophysitis. Further studies are needed to validate their pathogenic role and diagnostic utility.

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Introduction

Pituitary autoimmunity comprises a spectrum of clinical disorders and subclinical serological findings that include autoimmune hypophysitis,[1] isolated pituitary hormone deficiencies with pituitary antibodies,[2,3] and the pituitary antibodies found in patients with other autoimmune diseases such as thyroiditis.[4] Pituitary autoimmunity can be assessed by detecting pituitary autoantibodies, although this measurement has had limited clinical value mainly because the pathogenic pituitary autoantigens remain unknown.[5] Five candidates have been proposed through the years: GH in 2001,[6] alpha-enolase in 2002,[7] pituitary gland-specific factors 1a and 2 in 2002,[8] and more recently, in one patient, secretogranin II.[9]

GH was initially identified by Takao *et al.* from a nonapeptide sequence (FTPIPLSRL), corresponding to either the pituitary GH1 or the placental GH2. GH migrated as a 22-kDa band in one-dimensional (1D) immunoblots and was recognized by 11/15 (73%) patients with clinically suspected hypophysitis, 7/9 (78%) patients with isolated ACTH deficiency, but not by healthy controls, Hashimoto's thyroiditis or Graves' disease patients.[6] However, when Tanaka *et al.* expressed *in vitro* the full-length GH1, its recognition by hypophysitis sera was very low (2/17, 12%).[8]

Alpha-enolase was reported by Crock's laboratory from a 13-mer (GNPTVEVDLFTSK) detected by the serum of hypophysitis patients (who were previously shown to recognize a 49-kDa pituitary cytosolic band) but not by the normal serum.[7] However, when expressed *in vitro*, the full-length alpha-enolase was recognized not only by hypophysitis patients (7/17, 41%) but also by pituitary adenomas (6/13, 46%), other autoimmune diseases (6/30, 20%) and healthy controls (2/46, 4%),[10] weakening its clinical utility.

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Pituitary gland-specific factors 1a and 2 transcripts were found during a gene expression profile of the human pituitary,[11] but experimental support for the proteins is missing. They were expressed *in vitro* and reported to be recognized only minimally by hypophysitis sera, isolated ACTH deficiency, and other autoimmune diseases.[8]

The available pituitary autoantigens have not proven to be accurate as diagnostic markers or pathogenic in experimental animals, leaving us with only circumstantial evidence about their role as true autoantigens. Consequently, the development of antigen-based immunoassays, which is crucial for diagnostic or predictive purposes in many other autoimmune diseases,[12] has not occurred for hypophysitis. Such an immunoassay should improve the management of patients with suspected autoimmune hypophysitis who are often misdiagnosed as having a pituitary adenoma and undergo unnecessary transsphenoidal surgery.[13] The assay could, in fact, help to distinguish hypophysitis from other nonimmune, nonsecretory masses arising in the sella turcica.

The present study aimed to discover novel candidate pituitary autoantigens, using a proteomic approach (2D gel electrophoresis, immunoblotting and protein sequencing). We also compared immunofluorescence and 1D immunoblotting detection of pituitary antibodies for their accuracy in diagnosing autoimmune hypophysitis.

Subjects and methods

Subjects

This case–control study enrolled 28 hypophysitis cases and 98 controls. Cases included 19 women aged (mean ± SD) 31 ± 12 years, three diagnosed in the peripartum period, and nine men aged 42 ± 14 years, whose key clinical characteristics are summarized in Table 1. Cases were obtained from the following sources: 14 from Johns Hopkins (three from the hospital and 11 through our hypophysitis website), five from the National Cancer Institute, NIH, three from the University of Göttingen, Germany, three from the University of Cagliari, Italy, and three from Auckland, New Zealand. In 14 cases the diagnosis was established by histopathological examination of the pituitary specimen, which showed lymphocytic ($N = 10$) or granulomatous ($N = 4$) hypophysitis. The granulomatous cases were combined with the lymphocytic cases because their pituitary showed a lymphocytic infiltration accompanying the multinucleated giant cells (in the absence of tuberculosis, syphilis, sarcoidosis or Wegener granulomatosis), and it has been proposed that the two pathological forms represent phases of the same disease.[14,15] The remaining 14 cases were referred to us by endocrinologists who based the diagnosis of autoimmune hypophysitis on clinical and radiological criteria. This group of nonhistologically proven cases included five patients with advanced melanoma who developed hypophysitis while receiving infusions of a monoclonal antibody that blocks cytotoxic T-lymphocyte-associated antigen-4.[16]

Control individuals included pituitary adenomas ($N = 14$), autoimmune thyroiditis ($N = 48$), and healthy subjects ($N = 36$), all provided by the Department of Endocrinology, University of Pisa, Italy. Pituitary adenomas (six females and eight males, aged 50 ± 16 years) were sequentially recruited between May 2004 and February 2005 and included six nonsecreting, four GH-, two PRL-, one TSH- and one ACTH-secreting adenomas. Thyroiditis controls were chosen from a recently published cohort[4] based on a high prevalence (around 38%) of positivity for pituitary antibodies by immunofluorescence, and consisted of 15 Graves' disease patients (11 females and four males, aged 45 ± 16 years) and 33 Hashimoto's thyroiditis (32 females and one male, aged 40 ± 15 years). Healthy subjects (30 females and six males, aged 50 ± 13 years) were selected based on normal physical examination, normal endocrine function, and absence of thyroperoxidase or thyroglobulin autoantibodies (measured using the kit from ICN Pharmaceuticals, Inc., Brussels, Belgium). The study was approved by the institutional ethics committee, and informed consent was obtained from all participants.

1D gel electrophoresis of human pituitary cytosolic proteins and immunoblotting using human sera

Adult human pituitary glands were obtained within 18 h of death from the Johns Hopkins autopsy laboratory, frozen in liquid nitrogen, and stored until used. Glands were homogenized in phosphate-buffered saline (PBS) supplemented with a cocktail of protease inhibitors (Roche Applied Sciences, Indianapolis, IN). The homogenate was first centrifuged at 2000 *g* for 15 min at room temperature to remove the nuclear fraction and undisrupted debris, then at 100 000 *g* for 1 h at 4 °C to separate membrane (pellet) from cytosolic (supernatant) fractions. Cytosolic pituitary proteins were depleted of immunoglobulins using commercially available protein A/G agarose beads (Pierce, Rockford, IL), quantified by BCA (Pierce), loaded onto a 6-cm-wide preparative well (250 μg in 100 μl), and then fractionated onto 8–16% Tris-glycine polyacrylamide minigels (Invitrogen), under denaturing and reducing conditions. All minigels included a molecular weight standard, and were run at constant voltage (125 V), time (90 min) and temperature (25 °C).

Minigels were blotted onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ) that were then cut into 4-mm-wide strips. Individual strips were incubated overnight at 4 °C with sera (diluted 1 : 100 in PBS) randomly chosen using a stratified design that balanced the four different diagnostic categories in each minigel and included several repeats. Strips were finally incubated with an anti-human immunoglobulin G (IgG) antibody (Jackson Immunoresearch Laboratory, West Grove, PA) and a chemoluminescent substrate (GE Healthcare), following the manufacturer's recommendations.

Photographic films showing the binding of autoantibodies to pituitary proteins were scanned and analysed in Photoshop (Adobe Systems Incorporated, San Jose, CA) to measure the electrophoretic mobility (in mm) of all bands. Based on the mobility of the protein standards, a standard curve of molecular weights (in kDa) was constructed in each gel, by smoothing spline regression.[17] From the gel-specific standard curves, we derived the molecular weight of all bands in each strip, using R statistical software, version 2·5.[18]

Statistical analysis of the molecular weights

A first analysis of the pituitary immunoblots revealed that each serum recognized a large number of bands (Fig. 1). Possible

Table 1. Clinical features of 28 patients with autoimmune hypophysitis. Patients 1–14 are histologically proven, patients 15–28 clinically suspected

Patient no.	Sex, age	Clinical presentation	Endocrine defects at diagnosis	MRI findings	Pathology	Related to pregnancy	Autoimmune thyroiditis	Concurrent treatment	27 kDa band
1	F, 28	Headache, amenorrhoea	Hyperprolactinaemia	uk	LH	No	No	uk	No
2	M, 34	Headache, loss of libido	uk	uk	LH	NA	No	uk	No
3	F, 35	uk	uk	uk	LH	uk	Yes	uk	No
4	F, 34	Headache, visual field defect	uk	uk	LH	Yes	No	uk	No
5	F, 62	Headache, asthenia	Hypopituitarism	Sellar-suprasellar cystic mass	LH	No	Yes	Glucocorticoid	Yes
6	F, 40	Headache, asthenia	Hypocortisolism	Sellar mass	LH	uk	No	uk	Yes
7	F, 33	Headache, visual field defect		Sellar-suprasellar mass	LH	Yes	Yes	uk	Yes
8	M, 51	uk	uk	uk	LH	NA	No	uk	Yes
9	F, 28	Headache, amenorrhoea	uk	uk	GH	No	No	uk	Yes
10	F, 31	Amenorrhoea, galactorrhoea	Hyperprolactinaemia	uk	GH	No	Yes	uk	No
11	F, 43	Headache	Hypopituitarism	uk	GH	No	No	uk	Yes
12	M, 34	Polyuria, polydipsia, headache, loss of libido	Hypopituitarism, diabetes insipidus	Sellar-suprasellar cystic mass	GH	NA	No	uk	Yes
13	F, 11	Polyuria, polydipsia, visual field defect, growth retardation	Hypopituitarism, diabetes insipidus	Infundibular mass/atrophy of neurohypophysis	LH secondary to germinoma	No	No	uk	Yes
14	F, 22	Asthenia	uk	uk	LH	Yes		Glucocorticoid	Yes
15	F, 56	Polyuria, polydipsia	Diabetes insipidus, hypogonadism	Infundibulum diffusely thickened	ND	No	No	uk	No
16	F, 38	uk	Central hypothyroidism	Pituitary enlargement	ND	No	No	uk	No
17	M, 50	uk	Hypopituitarism	Pituitary enlargement	ND	NA	No	Anti-CTLA4	No
18	M, 49	uk	Hypopituitarism	Pituitary enlargement	ND	NA	No	Anti-CTLA4	No
19	M, 51	uk	Hypopituitarism	Pituitary enlargement	ND	NA	No	Anti-CTLA4	Yes
20	M, 52	uk	Hypopituitarism	Pituitary enlargement	ND	NA	No	Anti-CTLA4	Yes
21	M, 48	uk	Hypopituitarism	Pituitary enlargement	ND	NA	No	Anti-CTLA4	No
22	F, 24	Asthenia	Central hypothyroidism, hypoadrenalism	uk	ND	uk	No	uk	Yes
23	F, 26	Polyuria, polydipsia, amenorrhoea postpartum	Diabetes insipidus, hypogonadism	uk	ND	Yes	No	uk	Yes
24	F, 38	Polyuria, polydipsia	Diabetes insipidus	Infundibular and suprasellar mass	ND	No	No	uk	Yes
25	F, 33	uk	uk	Pituitary enlargement	ND	uk	No	None	No
26	M, 39	Headache	Isolated GH defect	uk	ND	uk	No	uk	Yes
27	F, 35	Asthenia	uk	uk	ND	No	No	None	No
28	M, 50	Asthenia	Hypogonadism	Pituitary atrophy, empty sella	ND	NA	Yes	None	Yes

NA, not applicable; ND, not done; uk, unknown.

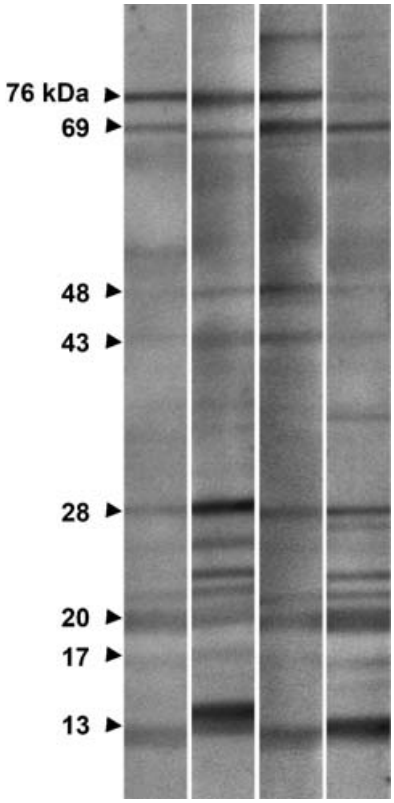

Fig. 1 1D gel electrophoresis and immunoblotting. Human pituitary cytosolic proteins were fractionated on polyacrylamide gels and then tested with sera from healthy controls (lane 1), pituitary adenomas (lane 2), autoimmune thyroiditis (lane 3) or autoimmune hypophysitis (lane 4). Arrowheads indicate the bands common to all lanes.

differences between cases and controls could not be identified by simple visual inspection. We therefore first subtracted the bands that were present in every strip of each gel (Fig. 1, arrowheads), and then used the following statistical approach to identify bands that were over-represented in individual disease categories. We analysed a pair of categories at a time, using a sliding window across the spectrum of molecular weights with a fixed width of 0·04. This value corresponds approximately to the error occurring when measuring band mobilities. Within each window, we formed a 2×2 table classifying the subjects in the two disease categories according to the presence or absence of a band within that window. We then formed a likelihood ratio test (LRT) statistics to compare the frequencies of bands in that particular window between the two disease categories. The LRT statistics was 'signed', so that a negative value indicated that a particular band was more common in the hypophysitis cases whereas a positive value indicated that the band was more common in the control group.

A large LRT value suggested a significant difference. We then used a permutation test to determine whether a particular LRT value was indeed statistically significant. In particular, we shuffled the data permuting the disease status of the subjects relative to the band size information, and keeping the band sizes for a particular subject together. With these shuffled data, the maximum LRT across all windows was calculated. We performed 100 000 such permutations to derive *P*-values, adjusting for the scan across windows. These *P*-values represented the proportion of permutations that gave a maximum LRT greater than or equal to the maximum LRT for the observed data.

Immunofluorescence to detect pituitary antibodies, and comparison with immunoblotting

Pituitary antibodies were measured by immunofluorescence in 74 subjects (all 28 hypophysitis cases, 14 pituitary adenomas, and 36 healthy subjects) using a commercially available kit (Euroimmun LLC, Boonton, NJ) based on *Macaca mulatta* pituitaries and read on a Zeiss fluorescence microscope by one author (I.L.). Immunofluorescence for the remaining 48 autoimmune thyroiditis patients was performed in a previous study,[4] using the same kit and reader (I.L.), which yielded five positive and 10 negative results for the 15 Graves' disease patients, and 12 positive and 21 negative for the 33 Hashimoto's thyroiditis patients. The accuracy of immunofluorescence and 1D immunoblotting in diagnosing autoimmune hypophysitis was quantified in terms of sensitivity (ability of the test to identify correctly those who have the disease), specificity (correct identification of those who do not have the disease), and area under the Receiver Operating Characteristic (ROC) curves.

Identification of pituitary autoantigens by 2D gel electrophoresis, immunoblotting and mass spectrometry

Pituitary cytosolic proteins were precipitated by 10% trichloroacetic acid/acetone, and redissolved in 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl (pH 8·8). Proteins (600 μg) were loaded onto 24-cm-long 'immobilized pH gradient' (IPG) strips (GE Healthcare) in isoelectric focusing buffer (8 M urea, 4% CHAPS, 0·2% DTT, 1·5% IPG buffer, 0·002% bromophenol blue), and separated by 10 h at 20 V, 30 min at 500 V, 30 min at 1000 V, and 4·5 h at 3000 V. A pair of identical pH 4–7 IPG strips was used in each experiment. After the first dimension, strips were cut into three fragments, each representing one pH unit, and run on 4–12% minigels for the second dimension. From the pair of identical strips, one minigel was stained by Coomassie blue, the other was transferred to nitrocellulose membranes. Membranes were blocked in PBS–3% bovine serum albumin, incubated overnight at 4 °C with diluted serum (1 : 100) from healthy controls, washed, and incubated for 1 h at room temperature with an anti-human IgG antibody, conjugated to horseradish peroxidase. Antibody binding was then detected using a chemoluminescent substrate. The membranes were then stripped, according to the manufacturer's recommendation (GE Healthcare), and probed with hypophysitis sera. Images produced by cases were compared to those produced by healthy controls to identify unique protein spots. These spots were then matched to the gels stained by Coomassie blue, excised, in-gel digested with trypsin, and submitted to matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry sequencing at the Proteomics Facility of the Johns Hopkins School of Medicine, as described previously.[19] The mass spectrometry peptides were used to query the 'all species' database of primary protein sequences using

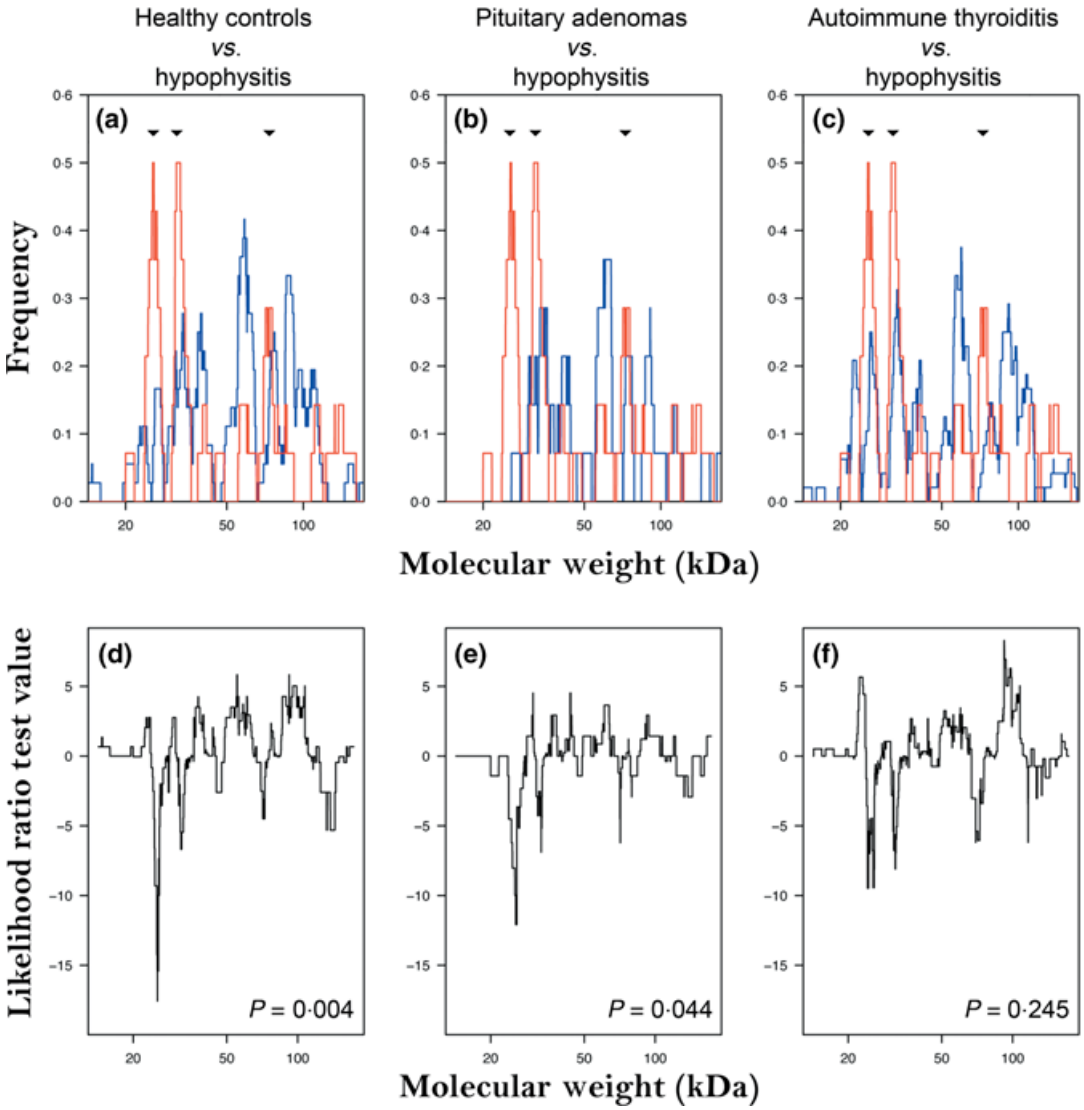

Fig. 2 Recognition frequency of human pituitary cytosolic proteins. Hypophysitis cases are compared with healthy controls (a and d), pituitary adenomas (b and e), and thyroiditis (c and f). (a–c) The proportion at which a particular molecular weight band is recognized in the pairwise comparisons (hypophysitis cases are the red lines, controls the blue lines). (d–f) The values of the likelihood ratio test in these comparisons.

Mascot search engine. Accuracy in protein identification is summarized in the Mowse score, which expresses the confidence with which a peptide sequence is correctly assigned to a protein by the search engine.[20] Scores ≥ 50 were considered as positive identifications. Scores do not reflect the abundance of the protein because mass spectrometry, under these experimental conditions, is not quantitative.[21]

Results

Pituitary cytosolic proteins are strongly recognized by human sera

All sera of both cases and controls bound numerous pituitary cytosolic antigens spanning the entire molecular weight spectrum (Fig. 1). In particular, the bands that were common to the entire study population corresponded to molecular weights of 13, 17, 20, 28, 43, 48, 69 and 76 kDa (Fig. 1, arrowheads). Based on the simple visual analysis, it was impossible to identify a particular band, or banding pattern, typical of a given disease category. We therefore opted for a comprehensive statistical analysis, which proved more revealing.

Pituitary cytosolic proteins in the 25–27-kDa range were preferentially recognized by autoimmune hypophysitis. The statistical analysis revealed three molecular weight ranges that were recognized more frequently by hypophysitis cases (red lines in Fig. 2) than by controls (blue lines). These regions were 25–27, 32–34 and 72–76 kDa (Fig. 2a,b,c, arrowheads). Only the 25–27-kDa region, however, reached statistical significance. This region was detected by 14/28 (50%) hypophysitis and 0/14 (0%) pituitary adenomas,

Table 2. Pituitary antibody results in the 126 study subjects. Antibodies were measured by Western blot, assessing the presence of a band in the 25–27-kDa region of human pituitary cytosolic proteins, and by immunofluorescence on *Macaca mulatta* pituitary sections

	25–27-kDa band present	Immunofluorescence positive
Healthy controls	3 of 36 (8%)	1 of 36 (3%)
Pituitary adenomas	0 of 14 (0%)	1 of 14 (7%)
Autoimmune thyroiditis	9 of 48 (19%)	17 of 48 (35%)
Graves' disease	2 of 15 (13%)	5 of 15 (33%)
Hashimoto's thyroiditis	7 of 33 (21%)	12 of 33 (36%)
Autoimmune hypophysitis	14 of 28 (50%)	16 of 28 (57%)
Histologically proven	9 of 14 (64%)	8 of 14 (57%)
Clinically suspected	5 of 14 (36%)	8 of 14 (57%)

Table 3. Accuracy of Western blot (WB, 25–27-kDa band) and immunofluorescence (IF) pituitary antibodies in diagnosing hypophysitis. The analysis was performed using as cases either the 14 histologically proven hypophysitis patients or all of the 28 hypophysitis (histologically proven and not) patients

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
WB_{histo}	64	88	43	95	0·7455
IF_{histo}	57	81	30	93	0·6652
WB_{all}	50	88	54	86	0·6888
IF_{all}	57	81	46	87	0·6888

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the receiver operating characteristic (ROC) curve.

9/48 (19%) selected autoimmune thyroiditis and 3/36 (8%) healthy controls ($P < 0·001$ by the χ^2 test, Table 2). Recognition in the cases was higher when only the histologically proven patients were included (9/14, 64%, Table 2). Males and females did not differ in the recognition pattern, but two of the three histologically proven hypophysitis women who were pregnant indeed recognized the 25–27-kDa region (data not shown).

Pairwise comparisons of hypophysitis cases *vs.* controls showed LRT values of –17·7 with healthy controls ($P = 0·004$, Fig. 2d) and –12 with pituitary adenomas ($P = 0·044$, Fig. 2e). However, no difference was observed with the autoimmune thyroiditis (LRT = –9·4, $P = 0·245$, Fig. 2f). This probably reflects our selection bias, considering that thyroiditis sera were chosen based on their immunofluorescence positivity, although a true comorbidity between these two diseases cannot be excluded.

When re-examined in light of the information provided by the statistical analysis, a distinct band in the 25–27-kDa region could indeed be seen, migrating just anodally from the 28-kDa common band (Fig. 3a).

Immunoblotting tends to be better than immunofluorescence in detecting pituitary antibodies

Pituitary antibodies detected by the presence of a band in the 25–27-kDa region in immunoblots were found in 9/14 histologically proven hypophysitis cases and 12/98 controls: 3/36 healthy controls, 0/14 pituitary adenomas and 9/48 autoimmune thyroiditis (Table 2). These values yielded a sensitivity of 64% and a specificity of 88% (Table 3). On the contrary, pituitary antibodies measured by immunofluorescence were positive in 8/14 histologically proven hypophysitis cases and 19/98 controls (1/36 healthy subjects, 1/14 pituitary adenomas and 17/48 autoimmune thyroiditis, Table 2), yielding 57% sensitivity and 81% specificity (Table 3). The immunofluorescence staining pattern was similar in cases and controls (data not shown).

The area under the ROC curve for immunoblotting was greater (0·7455) than that for immunofluorescence (0·6652, Table 3), but not significantly greater ($P = 0·347$). Overall, current immunoblots and immunofluorescence methods do not appear to have enough accuracy to make them useful in the diagnosis of autoimmune hypophysitis.

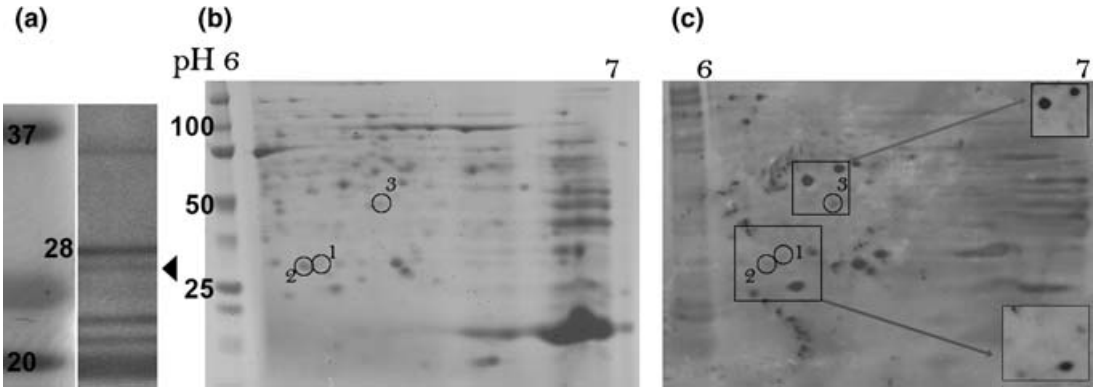

Fig. 3 Immunoblotting of human pituitary cytosolic proteins. (a) A 1D gel immunoblot using hypophysitis serum, focusing on the uniquely recognized band around 27 kDa. (b) A 2D gel in the pH 6–7 range stained with Coomassie blue, indicating the three protein spots that were selected for mass spectrometry sequencing. (c) The immunoblot of the gel shown in (b) using hypophysitis serum. The insets in the upper and lower right corners indicate the results obtained for the same gel region using healthy control serum.

Table 4. Mass spectrometry sequencing results, showing amino acid sequence and key features of the peptides identified following trypsin digestion of pituitary cytosolic spots 1, 2 and 3. The spot location is indicated in Fig. 3b,c. Spots 1 and 2 were uniquely recognized by hypophysitis sera, whereas spot 3 was recognized by both hypophysitis and, although with lower strength, normal sera. Spot 3 was included as an internal control for the sequencing because it was predicted to correspond to alpha-enolase

Spot	Tryptic peptide(s)	Score	Protein name	ID	MW (kDa)	Protein location	Protein function
1,2	KINEAIVAVQAIIADPKT and KNAEPLINLDVNNPDFKA	398	Chromosome 14 open reading frame 166 (aka CGI-99 or CLE)	AAV54070	28	Mainly in cytoplasm but also in nucleus	RNA binding protein
1	KDLEEGIQTLMGRL	54	Chorionic somatomammotrophin, or GH1	AAT11508, or AAH62775	25	Cytosol and granules	Promotes linear growth, influences protein, lipid, and sugar metabolism
1	RVLVEPDAGAGVAVMK and RDADVQNFVSFISKD	180	*Cis* delta 3, *trans* delta 2 enoyl coenzyme A isomerase	AAB32979	29	Mitochondria	Catalyses the conversion of the double bond between C3 and C4 during the degradation of unsaturated fatty acids
1,2	RYADLTEDQLPSCESLKD and RDAGYEFDICFTSVQK	169	Phosphoglycerate mutase	AAH00455	28	Cytosol	Catalyses the shift of the phosphoryl group from oxygen 3 to oxygen 2 within phosphoglycerate, as part of the glycolysis
1,2	KVTNGAFTGEISPGMIKD, RIIYGGSVTGATCKE and KSNVSDAVAQSTRI	143	Triose phosphate isomerase	EAW88721	27	Cytosol	Catalyses the reversible conversion of glyceraldehyde 3-phosphate (an aldose) to dihydroxyacetone phosphate (a ketose), as part of glycolysis
1,2	KIFEEDPAVGAIVLTGGDKA	125	Enoyl coenzyme A hydratase	AAH08906	31	Mitochondria	Catalyses the hydration of the double bond between C2 and C3 during the degradation of saturated fatty acids
1	RDPDAQPGGELMLGGTDSKY	93	Cathepsin D preproprotein	NP_001900	45	Lysosomes	Cleaves proteins at aspartic acid residues
3	KDATNVGDEGGFAPNILENKE and KVVIGMDVAASEFFRS	288	Enolase 1 (alpha)	AAB88178	49	Cytosol	Catalyses the reversible dehydrationof 2-phosphoglycerate into phosphoenolpyruvate, as part of glycolysis and gluconeogenesis

Identification of pituitary autoantigens by 2D-gel electrophoresis, immunoblotting, and mass spectrometry

To better resolve the pituitary cytosolic protein(s) contained in the 25–27-kDa region, we performed 2D gel electrophoresis of human pituitary cytosolic proteins, followed by immunoblotting with control sera and then hypophysitis sera. The 25–27-kDa band separated into two closely juxtaposed protein spots in the pH 6–7 range (Fig. 3b, spots 1 and 2). These spots were uniquely recognized by hypophysitis cases (Fig. 3c), and therefore excised for mass spectrometry sequencing. A spot around 49 kDa, recognized by hypophysitis cases (Fig. 3c, spot 3) but also at lower intensity by healthy controls (Fig. 3c, top right inset), was chosen for sequencing as an experimental control, because it was predicted to correspond to the previously identified alpha-enolase.[7] Sequencing of the excised spots identified the candidate pituitary autoantigens listed in Table 4.

C14orf166 emerged as a novel pituitary autoantigen, with the strongest identification score (Table 4). Little is known about the function of this protein, which has not been previously reported in the pituitary.

Numerous housekeeping proteins (coenzyme A isomerase and hydratase, phosphoglycerate mutase, triose phosphate isomerase and cathepsin D) were also identified, a common proteomic finding considering that these proteins are typically over-represented in heterogeneous protein databases.[22]

Interestingly, the human chorionic somatomammotrophin hormone (CSH) appeared as an attractive candidate autoantigen. This peptide sequence corresponds to the isoform 1 of CSH1 or CSH2, two placental proteins of 217 amino acids in the GH gene family that are 99% identical (differing only at their third residue). The sequence is also found in the pituitary GH1, isoforms 1, 2 and 3 (Fig. 4), revealing a link for a pituitary disease such as autoimmune hypophysitis that typically manifests during pregnancy.

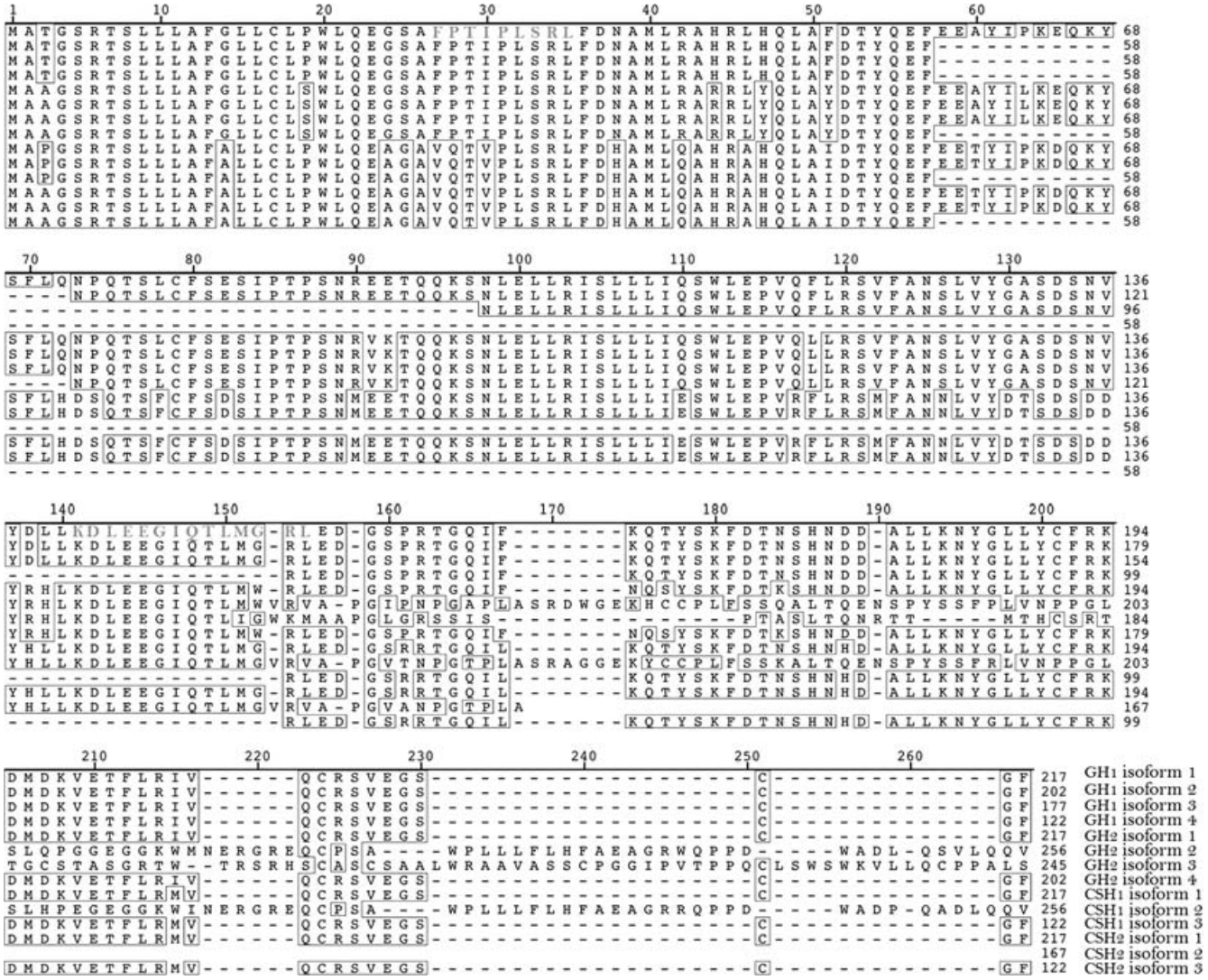

Fig. 4 Alignment of the proteins in the GH gene family. The four isoforms of pituitary GH1, the four isoforms of placental GH2, and the three isoforms of the two chorionic somatomammotrophins are shown. The short isoform 5 of GH1 (mateagrwqppdwadlqadlqqvrhkltqr) was removed for clarity.

Discussion

This paper reports the identification of novel pituitary autoantigens in a case–control study comparing autoantibodies from autoimmune hypophysitis cases with those from thyroiditis, pituitary adenoma and healthy controls. These autoantigens migrated in the 27-kDa region and included two notable proteins: C14orf166 and chorionic somatomammotrophin.

C14orf166 is encoded by a gene located on the long arm of chromosome 14 that is highly conserved among species. It localizes in both the cytosol[23] and the nucleus,[24,25] and has been reported in the mouse brain[26,27] and human endothelium,[28] as well as in various human cell lines: HeLa cervical cancer,[23,24,29] RF-1 gastric adenocarcinoma,[30] DG75 Burkitt's lymphoma[31] and Daoy medulloblastoma.[32] The function of C14orf166 remains to be defined. It interacts with RNA polymerase of the influenza A virus[23] and prevents phosphorylation of ninein,[25] suggesting a role in the regulation of centrosome structure. Very recently, Wang *et al*. reported that C14orf166 is down-regulated during mouse brain development, from embryonic day 16 to postnatal day 56.[27] Very little is known about a possible role of C14orf166 as an autoantigen. One study showed that C14orf166 is immunogenic because it was recognized, albeit at low frequency, by one of 20 sera from hepatocellular carcinoma cases and not by 16 chronic viral hepatitis or 20 healthy controls.[33] It remains to be shown whether C14orf166 is indeed a true pathogenic autoantigen in autoimmune hypophysitis.

The other interesting sequence identified by this study is KDLEEGIQTLMGRL. This sequence is shared among GH1 (isoforms 1, 2 and 3), CSH-1 (isoform 1) and CSH-2 (isoform 1) (Fig. 4). The human GH family includes four expressed, highly similar, genes (named GH-N, GH-V, CS-A and CS-B) located in close proximity on the long arm of chromosome 17. The N gene encodes GH1, which is expressed primarily in the pituitary gland and has five isoforms, originated by alternative splicing. The V gene is

expressed in the placenta and produces GH2, which has four isoforms. The chorionic somatomammotrophin A and B genes are expressed in the placenta and produce two almost identical proteins, named CSH-1 and CSH-2, each having three isoforms.

It is intriguing to speculate on the possible role of our sequence as a pituitary autoantigen. GH was indeed the first protein to be proposed as an autoantigen in autoimmune hypophysitis, from the sequence FPTIPLSRL.[6] This sequence, however, is extremely conserved and it is found not only in GH1 (isoforms 1–4) but also in GH2 (all isoforms, Fig. 4). Our results identify a different region in the GH family, shared by pituitary GH1 and placental CSHs. These findings provide a possible mechanism for the striking temporal association between autoimmune hypophysitis and pregnancy.[1] An immune reaction against that particular placental CSH epitope could spread to the pituitary recognizing the same epitope in GH1, an example of what is known in immunology as molecular mimicry.[34] Notably, there is circumstantial evidence that CSH can indeed be a true pathogenic autoantigen in hypophysitis. Beck and Melvin were, in fact, capable of inducing experimental hypophysitis in one female rhesus monkey by injecting her with human placental extracts and chorionic somatomammotrophin.[35]

The five pituitary enzymes revealed by our mass spectrometry sequencing are ubiquitous and not pituitary specific. It is seemingly difficult to reconcile how a ubiquitous antigen could be implicated in the pathogenesis of an organ-specific autoimmune disease such as hypophysitis. The only example that comes to mind is that of glucose-6-phosphate isomerase, a glycolytic enzyme that, despite its universal expression, induces in mice a joint-specific disease when targeted by autoimmunity.[36] A possible interpretation of this apparent paradox is that ubiquitous autoantigens are pathogenic only during the maintenance or progression stages of the disease, rather than during the initiation. An elegant experimental demonstration of this postulate comes from the mouse model of multiple sclerosis, known as experimental autoimmune encephalomyelitis. Investigators have been able to induce demyelination and weakness of the limbs by immunizing mice with myelin basic protein, myelin proteolipid protein or myelin oligodendroglial protein, therefore fulfilling Witebsky's postulates[37] of pathogenicity. In patients, however, autoreactive B cells and T cells recognize predominantly αB-crystallin,[38] a small heat-shock protein expressed in skeletal and heart muscles and glial cells. Discouragingly, immunization of mice with αB-crystallin was not able to induce encephalomyelitis.[39] Recently, Ousman *et al.* have shown that αB-crystallin becomes an autoimmune target after the disease has been initiated by myelin oligodendroglial glycoprotein immunization, and its destruction worsens demyelination, although it does not directly cause it.[40]

Finally, our study compared for the first time, in a large patient population of hypophysitis cases and controls, the methods of immunoblotting and immunofluorescence for detecting pituitary antibodies. Immune recognition of the 25–27-kDa region tended to be more sensitive and specific for hypophysitis than immunofluorescence, although both methods do not currently allow autoimmune hypophysitis to be distinguished from other pituitary lesions or other autoimmune conditions and require validation in a larger number of patients. Identification of the pathogenic pituitary antigen(s) will lead to the development of antigen-specific immunoassays, which should strengthen our clinical armamentarium and elucidate the clinical significance of pituitary antibodies.

In summary, we have reported the proteomic map of human pituitary cytosolic proteins recognized by sera from patients with autoimmune hypophysitis. Validation of the novel candidate autoantigens identified here is the next essential step to advance our understanding of pituitary autoimmunity and design antigen-based diagnostic tests.

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