

CLINICAL STUDY

Prevalence of calcium sensing receptor autoantibodies in patients with sporadic idiopathic hypoparathyroidism

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Abstract

Objective: The pathogenesis of sporadic idiopathic hypoparathyroidism is unclear. The calcium sensing receptor (CaSR) plays a pivotal role in extracellular calcium homeostasis and is the candidate autoantigen in hypoparathyroidism associated with autoimmune polyglandular endocrinopathy syndrome. We therefore looked for antibodies (Ab) against the CaSR in patients with sporadic idiopathic hypoparathyroidism and their association, if any, with the major histocompatibility complex (MHC) class II human leukocyte antigen (HLA)-DR haplotypes.

Methods: The subjects included 51 patients with sporadic idiopathic hypoparathyroidism and 45 healthy controls. Investigations included computerised tomography, serum calcium, phosphorus, thyroxine, TSH, cortisol, intact parathyroid hormone (iPTH), ACTH and thyroid peroxidase (TPO) and adrenal antibodies. The CaSRAb were assayed in patients' sera by Western blot. Genotyping of the HLA-DR locus was performed using PCR and sequence-specific oligonucleotide probes.

Results: Intracranial calcification and cataract were present in 76.5% and 41.1% of the patients respectively and 62.7% had convulsions. Autoantibodies against the 168 kDa CaSR protein were demonstrated in the serum of 49.0% of the patients and in 13.3% of the controls ($P < 0.001$). Pre-incubating serum samples from the CaSRAb-positive patients with parathyroid membrane produced a 90% decrease in the band intensity. HLA-DRB1*01 and DRB1*09 alleles were significantly associated with idiopathic hypoparathyroidism (relative risk of 7.8, $P = 0.001$). The frequency of HLA-DRB1*09 and DRB1*10 alleles tended to be higher in patients positive for the CaSRAb. There was no significant difference in the frequency of occurrence of convulsions, cataract, intracranial calcification, calcium:phosphorus ratio, and iPTH levels between patients with and without CaSRAb.

Conclusion: 49.0% of the patients studied had serological evidence of organ-specific autoimmunity against the CaSR protein. The occurrence of CaSRAb and the HLA-DR associations imply an autoimmune component to the disease, but the primary role of the CaSRAb in the pathogenesis of the disease needs to be assessed further.

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Introduction

Idiopathic hypoparathyroidism is a heterogeneous group of disorders clinically characterised by the presence of tetany, cataract and basal ganglia calcification in association with hypocalcaemia, hyperphosphataemia and low or inappropriately normal serum intact parathyroid hormone (iPTH) (1–5). The pathogenesis of sporadic idiopathic hypoparathyroidism has not yet been explained. In 1966, Blizzard *et al.* using indirect immunofluorescence, had reported the presence of parathyroid autoantibodies in patients with idiopathic hypoparathyroidism, but the organ-specific nature of these antibodies has been a subject of controversy

(6–9). Recently, the calcium sensing receptor (CaSR), which is a G-protein-coupled receptor, has been shown to play a pivotal role in the maintenance of the extracellular calcium homeostasis, which it does by sensing the circulating calcium levels and regulating the PTH synthesis and release (10–13). Li *et al.* have reported the presence of autoantibodies against the CaSR among patients with hypoparathyroidism, in the majority of whom it was associated with the type 1 autoimmune polyglandular syndrome (APS-1) (14). However, to our knowledge there is no report that has investigated patients with sporadic idiopathic hypoparathyroidism for the presence of similar autoantibodies. Therefore, we studied the prevalence of CaSR

autoantibodies (CaSRAb) in a group of patients with sporadic idiopathic hypoparathyroidism without associated APS-1 and examined their association with the major histocompatibility complex (MHC) class II human leukocyte antigen (HLA)-DR and with the clinical and biochemical indices of the disease.

Subjects and methods

Subjects

The subjects included 51 consecutive patients with hypoparathyroidism (29 males, 22 females) who attended the Endocrine Clinic at the All India Institute of Medical Sciences (AIIMS), New Delhi, from 1998 to 2002. Their mean age at presentation was 33.1 ± 12.8 years and the mean duration of their illness was 7.0 ± 8.4 years. The diagnosis of idiopathic hypoparathyroidism was based on the presence of hypocalcaemia and hyperphosphataemia in association with an inappropriately normal or subnormal level of serum iPTH. Patients with post-surgical hypoparathyroidism were excluded. None of the patients had a family history suggestive of hypoparathyroidism or adrenal insufficiency or clinical features of mucocutaneous candidiasis. The type 1 autoimmune polyglandular syndrome was excluded by demonstrating a normal adrenocorticotrophin (ACTH) value in all the 38 of 51 patients in whom fresh plasma samples could be obtained for ACTH assay. The 0800 h serum cortisol was also normal in all the subjects and there were no adrenal cortical autoantibodies observed using the indirect immunofluorescence technique described earlier (15). Six of the 51 patients (11.7%; 5 females and 1 male) had coexistent autoimmune disorders as evidenced by overt hypothyroidism in three, type 1 diabetes mellitus in one, alopecia areata with thyroid peroxidase antibody (TPOAb) positivity in one and TPOAb positivity alone in one. The study was approved by the Research and Ethics Committee of the AIIMS and written informed consent for participation was obtained from all the subjects. Clinical symptoms, including the duration of the illness and the presence of tetany, convulsions, and cataract were noted. Non-contrast computerised tomography of the brain was carried out for intracranial calcification. Details of one patient with reversible hypocalcaemic peripheral neuropathy have been published earlier (16). A basal blood sample was drawn after an overnight fast (0800 h) for the estimation of serum calcium and phosphorus and for the assay of iPTH (DiaSorin, Inc., Stillwater, MN, USA), total thyroxine (T_4), thyrotrophin (TSH) (Medicorp Inc., Montreal, Quebec, Canada), cortisol (Immunotech, Marseille, France) and TPOAb (Immunotech, Prague, Czech Republic), plasma ACTH (DiaSorin, Minnesota, USA), as well as for CaSRAb assays. Serum calcium and phosphorus levels were measured using the standard techniques used in our

laboratory (17). All the samples were assayed for hormones in a single batch. The controls included 45 healthy subjects (26 males, 19 females, mean age 42 ± 15 years). The normal range for the basal hormone values were as follows: serum $T_4 = 52-167$ nmol/l, TSH = 0.3–4.0 mU/l, cortisol = 260–720 nmol/l, iPTH = 13–54 ng/l, ACTH = 1.3–12.4 pmol/l. Intra-assay coefficients of variation ranged from 4% to 8% for the measurement of these hormones. As specified by the manufacturers of the TPOAb-RIA kit, a serum TPOAb titre >100 IU/ml was considered positive.

Western blot for the CaSRAb

The presence of antibodies against the calcium sensing receptor was determined using Western blot analysis (11, 18). Briefly, parathyroid adenoma tissues were obtained at surgery from patients with primary hyperparathyroidism who underwent parathyroidectomy. The tissues were snap frozen in liquid N_2 and were homogenised in a glass homogeniser with a teflon pestle in ice-cold lysis buffer in a 1:2 ratio by volume of sample to lysis buffer (50 mmol/l Tris-HCl (pH 7.4); 0.25 mol/l sucrose; 1 mmol/l EGTA, EDTA, PMFS and 10 μ g/ml aprotinin, leupeptin and pepstatin (USB, Cleveland, OH, USA)). The nuclei and cell debris were pelleted by centrifugation at $800 \times g$ and the supernatant then recentrifuged at $15\,000 \times g$ to remove the mitochondrial fraction. The supernatant was finally sedimented by centrifugation at $45\,000 \times g$ for 1 h, and the pellet suspended in 2 ml 1% Triton X-100 lysis buffer. All the centrifugations were carried out at 4 °C. The protein content of the solubilised membrane fraction was estimated using Bradford's reagent and the readings taken at 595 nm (Shimadzu, UV160). Parathyroid adenomas have been reported to show a variable, often consistent reduction in the CaSR expression (19). Therefore, in this study we used only those parathyroid adenoma tissues whose cell membranes showed a relatively high level of expression of the CaSR for screening the patients' sera for the CaSRAb. This level of expression of the CaSR in the different adenoma membranes was assessed by first staining all our membrane preparations using a commercially available anti-calcium sensing receptor antibody on Western blot (PA1-934, Affinity BioReagents, Inc., Golden, CO, USA). Only those membrane preparations which displayed a CaSR protein band of good intensity on Western blot analysis were subsequently utilised. The membrane fractions thus prepared were made into aliquots of 100 μ l each (containing 2 μ g/ μ l protein) and were stored at -20 °C until used. For assay, one aliquot of the fraction prepared as described above was mixed in a ratio of 1:5 with 5 \times Laemmli buffer (0.32 Tris pH 6.8), 5% (w/v) SDS, 25% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 143 mmol/l β -mercaptoethanol (Sigma, St Louis, MO, USA), and

20 µg of the protein were loaded in each well for electrophoresis without prior boiling. The proteins were fractionated using SDS-PAGE mini gels (8 × 7 cm with 5% resolving and 4% stacking gels; Hoeffer, San Francisco, CA, USA), transferred to PVDF membranes and probed overnight with patients' sera (1:50 dilution with 1% Blotto/PBS) at 4 °C using a deca-probe incubation manifold as described earlier (18). Anti-human (1:1000 dilution) and anti-rabbit IgG conjugated with horseradish peroxidase (1:500 dilution, DAKO, Glostrup, Denmark) were used as second antibodies (incubation time, 1.5 h) and 3-amino-9-ethylcarbazole in 0.1 mol/l acetate buffer (pH 5.2) was used for optimal colour development (AEC, DAKO, Carpinteria, CA, USA). In addition to the patients' sera, each blot also included 4 lanes, 2 of these containing various positive controls, pre-stained protein molecular weight markers, and a negative control containing only 1% Blotto/PBS. The various positive controls used included (i) a commercially procured antibody against the CaSR (PA1-934), (ii) a polyclonal, affinity-purified, anti-CaSR antibody (code: 4637) raised in rabbits as described earlier (19); both the above antibodies recognised the 168 kDa as well as the 151 kDa isoforms of the CaSR, and (iii) serum from a patient with hypoparathyroidism which contained an antibody strongly positive for only the 168 kDa CaSR protein. In order to minimise the introduction of bias in the interpretation of results, the blots were read independently by two investigators, one of whom (EMB) was kept blind to the identity of the samples (see Fig. 1).

Specificity of the CaSR antibody in the patients' sera was confirmed by doing neutralisation experiments demonstrating the blocking of these antibodies using antigens obtained from two different sources. (i) A

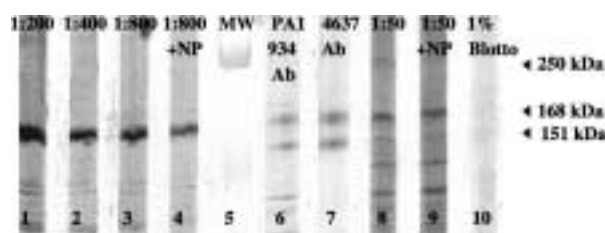


Figure 1 Immunoblotting of proteins from membranes prepared from human parathyroid adenomas. A 5% SDS-PAGE Western blot using parathyroid membrane protein (20 µg protein/lane) shows seropositivity against 168 and 151 kDa CaSR proteins by CaSR-antibody-positive control serum (PA1-934) in lane 6 and another positive control serum (4637 antibodies) in lane 7; negative control (1% blotto/PBS, blank) with no seropositivity against the 168 and 151 kDa proteins is shown in lane 10. A patient with hypoparathyroidism showing CaSRAb against 168 kDa in 1:200, 1:400 and 1:800 dilution (lanes 1, 2 and 3 respectively) and 28.4% neutralisation of seropositivity at a 1:800 dilution with neutralizing peptide (NP) PEP-009 (lane 4), and a second patient with hypoparathyroidism showing seropositivity in 1:50 dilution against 168 and 151 kDa proteins (lane 8) and 11.1% reduction of 151 kDa band positivity with NP-PEP-009 (lane 9) are also shown. Lane 5, molecular weight (MW) markers.

commercially available neutralizing peptide PEP-009, against which the PA1-934 antibody had been developed (Affinity BioReagents, Inc.); PEP-009 is a 16 amino acid synthetic peptide whose sequence is derived from and is identical to the amino terminal end of the rat CaSR protein (12–27 amino acids: A-L-A-W-H-S-S-A-Y-G-P-D-Q-R-A-Q). PEP-009 binds and blocks the rat as well as the human CaSRAb (20). (ii) A human parathyroid adenoma membrane preparation which showed a good expression of the CaSR on Western blot analysis as described above. Serum samples obtained from two patients with hypoparathyroidism, which were most strongly positive for autoantibodies against the 168 and 151 kDa CaSR proteins were first serially diluted to determine the lowest titre at which they tested positive for the CaSRAb. The neutralisation of these antibodies was then carried out by incubating the sera at this titre with 30 µg of the peptide PEP-009. The difference in the integrated density value of the CaSRAb band obtained in the presence and in the absence of the PEP-009 peptide was measured by densitometry using the image analyser software, AlphaEase (ChemImager 4400, Alpha Innotech Corp., San Leandro, CA, USA). In order to demonstrate specific neutralisation of the CaSRAb by the parathyroid adenoma membrane preparation, 10 µl serum from a patient whose serum was strongly positive for CaSRAb were first incubated overnight with an excess of the membrane protein on a rocker at 4 °C, before carrying out the Western blot analysis (200 µg membrane protein in 500 µl lysis buffer). The neutralisation was assessed by densitometry as described above. Similar neutralisation experiments to assess the specificity of the CaSRAb were also performed using membrane preparations (200 µg each) from human liver and skeletal muscle tissues obtained at autopsy (see Fig. 2B).

Western blot for CaSRAb using rat liver and myocardial tissue, as well as human liver and skeletal muscle membrane preparations

Since there are some reports in the literature showing weak expression of the CaSR in the rat hepatocyte and myocardial tissues (21, 22), we also examined these tissues along with human liver and skeletal muscle membranes, for expression of the calcium sensing receptor and to investigate their ability to detect the CaSRAb in the patients' sera (see Fig. 3A and B). The procedure adopted for extraction of membrane preparations from the above-mentioned tissues was essentially the same as described above for the parathyroid tissue membranes.

Genotyping for MHC class II, HLA-DR locus

In 36 of the 51 patients with hypoparathyroidism, DNA was extracted from 10 ml blood using the standard

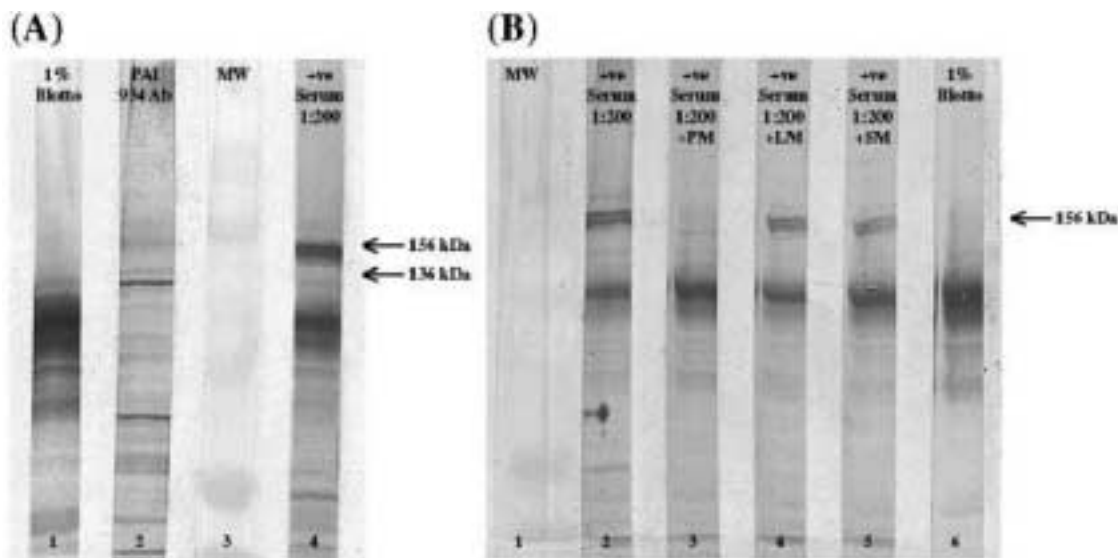


Figure 2 (A) Heterogeneity of the molecular weight of CaSR antigens. A 5% Western blot using parathyroid membrane protein (20 μ g protein/lane) shows CaSRAb against 156 kDa antigen in a patient with idiopathic hypoparathyroidism in a serum dilution of 1:200 in lane 4, and PA1-934 shows positivity against 156 and 136 kDa antigens in lane 2. Lane 1, blank and lane 3, molecular weight (MW) markers. The membrane preparation used in this experiment belonged to a different patient undergoing parathyroid adenectomy than that shown in Fig. 1. However, the sera in lane 4 of Fig. 2A, lane 2 of Fig. 2B and in lanes 1–3 of Fig. 1 were from the same patient. (B) Specific neutralisation of the CaSRAb in a patient with idiopathic hypoparathyroidism with parathyroid membrane protein. A 5% SDS-PAGE Western blot using various membrane protein preparations (20 μ g protein/lane) shows seropositivity against 156 kDa in a patient with hypoparathyroidism (1:200 dilution, lane 2) and 90% neutralisation of CaSRAb band positivity after overnight preincubation of the serum with parathyroid membrane (lane 3); only 16% and 28% neutralisation of CaSRAb positivity following preincubation of the CaSRAb-positive sera with cell membrane preparations from human liver (LM, lane 4) and human skeletal muscle (SM, lane 5) is seen. Lanes 1 and 6 are MW markers and blank (1% Blotto/PBS) respectively.

phenol–chloroform extraction technique (23). The HLA-DR genotyping was carried out using PCR (Applied Biosystem) followed by hybridisation with sequence-specific oligonucleotide probes (PCR-SSOP). Briefly, amplification of the second exon of the HLA-DRB1 gene was performed using the primers described earlier (24). The PCR products were run on 1% agarose gels to confirm the amplification and were then dot blotted on Hybond N(+) membranes (Amersham, Life Sciences Inc., Chicago IL, USA) and UV cross linked. The membranes with the dot blots were then hybridised with 32 P-labelled probes for DRB (1–18) generic. Differences in the frequency of the presence of specific HLA-DR alleles in patients with hypoparathyroidism as a group and when stratified according to CaSRAb positivity were compared with the data base already available to us from 94 healthy controls drawn from the same North Indian ethnic background (24).

Statistical analysis

Chi-square analysis was used to compare the frequency of occurrence of CaSRAb positivity between the patient and the control groups. The Chi-square test and the Student's *t*-test were used to compare the frequency of occurrence and the mean of the different variables respectively, between the group of patients with and without CaSRAb. A *P* value of <0.05 was considered

statistically significant. For HLA analysis, the *P* values were calculated using Fisher's exact test, and the results obtained were corrected by multiplying by the number of DR alleles tested. Relative risks and odds ratios were calculated using Woolf's method with Haldanes's modification (25).

Results

A history of convulsions, cataract and intracranial calcification on imaging was present in 32 (62.7%), 21 (41.1%) and 39 (76.5%) of the 51 patients respectively. Subjects with intracranial calcification had a higher frequency of occurrence of cataract when compared with those without calcification (19/39, 48.7% vs 2/12, 17.7%, *P* = 0.048). The mean duration of the illness was longer in patients who had intracranial calcification or cataract as compared with patients without these complications (9.0 ± 9.5 years vs 2.4 ± 4.1 years, *P* = 0.002 and 11.6 ± 10.4 years vs 4.4 ± 6.4 years, *P* = 0.01 respectively). On linear regression analysis of the data in models where age of onset of symptoms, duration of illness and serum calcium levels were considered as independent variables, only the duration of the illness explained the presence of the variations observed in the frequency of occurrence of cataract or basal ganglia calcification (dependent variables)

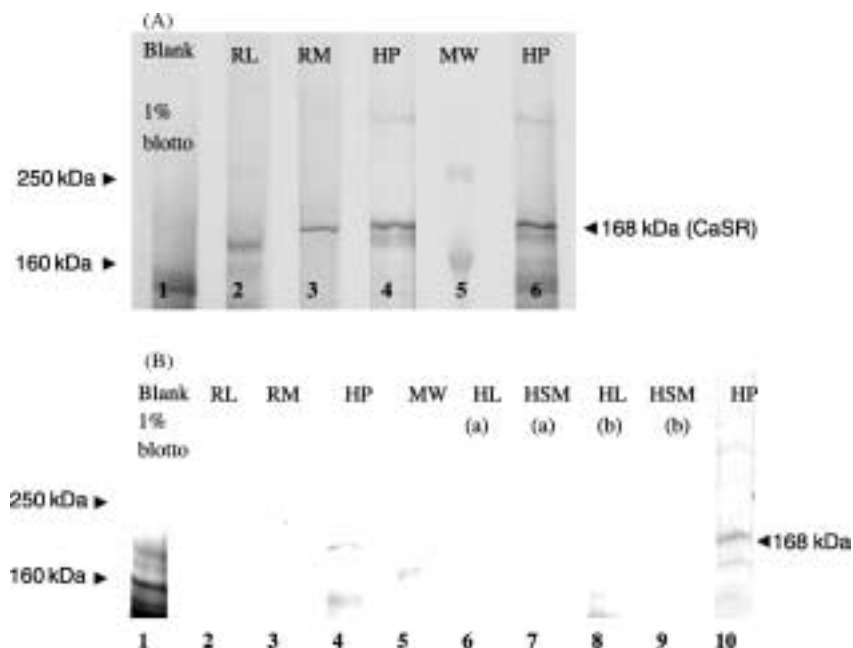


Figure 3 (A) Immunoblot of membrane proteins (20 μ g protein/lane) prepared from rat liver (RL, lane 2), rat myocardial tissue (RM, lane 3) and human parathyroid adenoma (HP, lanes 4 and 6). The 5% SDS-PAGE Western blot probed with the commercial CaSRAb-positive control serum (PA1-934) shows the presence of the 168 kDa CaSR protein in lanes 3 (RM) and 4 (HP) and is also positive for a 162 kDa antigen in lane 2 (RL). CaSRAb-positive serum (1:50 dilution) from a patient with hypoparathyroidism also shows reactivity against the 168 kDa CaSR protein in lane 6 (HP). Lanes 1 and 5 are blank (1% Blotto/PBS) and molecular weight (MW) markers respectively. (B) Immunoblot of membrane proteins (20 μ g protein/lane) prepared from rat liver (RL, lane 2), rat myocardial tissue (RM, lane 3), human parathyroid adenoma (HP, lanes 4 and 10), human liver and skeletal muscle tissue obtained from two different autopsies (HL a and b, lanes 6 and 8; HSM a and b, lanes 7 and 9). A 5% SDS-PAGE Western blot shows seropositivity against 168 kDa by PA1-934 CaSR antibody in lane 10. CaSRAb-positive serum (1:50 dilution) from a patient with hypoparathyroidism also shows positivity against the 168 kDa CaSR protein in lanes 4 (HP), 7 and 9 (HSM a and b respectively, weekly positive). The same patient's serum showed no seropositivity against CaSR protein in lanes 2 (RL), 3 (RM) and in lanes 6 and 8 (HL a and b respectively). Lanes 1 and 5 are blank (1% Blotto/PBS) and MW markers respectively.

respectively ($15.4 \pm 45.7\%$, $P < 0.01$ and 16.7 ± 40.2 , $P < 0.002$).

Western blot analysis demonstrated the presence of antibody against the CaSR in 25 of the 51 (49.0%) patients with hypoparathyroidism. The frequency of occurrence of the CaSRAb among the 45 subjects without any associated autoimmune endocrinopathy (51.1%) was similar to that observed for the whole group of 51 patients (49.0%). Twenty-one of the 25 patients showing CaSRAb were seropositive only for the 168 kDa protein while four were positive for both

the 168 as well as the 151 kDa protein (Table 1, Fig. 1). In contrast, only 6/45 (13.3%) of the controls studied had autoantibodies against the 168 kDa CaSR (patients vs controls $P < 0.001$). Following incubation with an excess of the synthetic neutralising peptide, PEP-009, whose sequence is identical to the amino terminal end of the rat CaSR protein and which also binds and blocks the human CaSRAb (20), there was a 28.4% and 11.1% reduction in the integrated density value (IDV) of the 168 kDa and 151 kDa bands respectively (Fig. 1, lane 3 vs lane 4 and lane 8 vs lane 9).

Table 1 Calcium sensing receptor and thyroid peroxidase autoantibodies status and HLA-DR associations in patients with idiopathic hypoparathyroidism and in healthy controls.

Parameters	Patients	Healthy controls	P value
Overall CaSRAb positivity	25/51 (49.0%)	6/45 (13.3%)	< 0.001
CaSRAb 168 kDa alone	21/51 (41.1%)	5/45 (11.1%)	0.0021
CaSRAb both 168 & 151 kDa	4/51	Nil	0.058
CaSRAb 151 kDa alone	Nil	Nil	—
Thyroid peroxidase antibody positivity	3 (5.8%)	0	0.24
Frequency of HLA-DRB1*01 allele	4/36 (11.1%)	1/94 (1.1%)	0.020
Frequency of HLA-DRB1*09 allele	4/36 (11.1%)	2/94 (2.1%)	0.049
Frequency of HLA-DRB1*01+DRB1*09 alleles	8/36 (22.2%)	3/94 (3.2%)	0.0015

Figure 2A and B displays the results of experiments showing specific neutralisation of the CaSRAB in a patient with idiopathic hypoparathyroidism. Following preincubation of the serum with an excess of parathyroid membrane proteins, the IDV of the CaSRAB band was reduced by 90% on blot densitometry. In contrast, the IDV of the CaSRAB band was reduced by only 16% and 28% respectively when liver and skeletal muscle membrane preparations were used. This reduction in IDV could be explained by the relatively weak expression of the CaSR in the human liver and skeletal muscle preparations (Fig. 3A and B). In the parathyroid adenoma membrane preparation used for neutralisation experiments, both the commercial anti-calcium sensing receptor antibody PA1-934, as well as the CaSRAB from the patient with hypoparathyroidism, reacted with the CaSR antigen protein at molecular weights corresponding to 156 kDa and 136 kDa and not with 168 kDa and 151 kDa, as observed in earlier preparations (Fig. 2A and B).

Both the rat liver and the rat myocardial membrane preparations showed CaSR expression as demonstrated by their reactivity with the PA1-934 CaSR specific antibodies (Fig. 3A). However, serum obtained from a patient with hypoparathyroidism, which had reacted strongly with the 168 kDa CaSR protein in a human parathyroid membrane preparation, did not react against the CaSR protein present in rat liver and myocardial membranes, possibly indicating the species-specific nature of the CaSRAB (Fig. 3B).

There was no significant difference in the frequency of occurrence of the TPOAb positivity between the patient and the control groups (Table 1). Table 2 gives the details of the various other indices related to hypoparathyroidism, which were also examined in patients with and without CaSRAB. Among the hypoparathyroid patients, there were no significant differences observed between the group of CaSRAB-positive and

CaSRAB-negative patients with regard to the male to female ratio, frequency of occurrence of tetany, convulsions, cataract, basal ganglia calcification, mean serum calcium, serum phosphorus or their ratios, serum iPTH values or the frequency of presence of TPOAb. The six subjects belonging to the control group who also demonstrated serological evidence of CaSRAB were asymptomatic and had no clinical or biochemical evidence of hypoparathyroidism.

Of the 18 HLA-DRB alleles analysed, the frequency of occurrence of HLA-DRB1*01 or DRB1*09 ($n = 4$ each) was significantly higher among the 36 patients with hypoparathyroidism when compared with the frequency of occurrence of the same alleles ($n = 1$ and 2 respectively) in the 94 healthy controls studied who were derived from the same ethnic background ($P = 0.001$, P corrected for 18 alleles tested = 0.02) with Haldanes' corrected relative risk of 7.8. The frequency of occurrence of the HLA-DRB1*09 or DRB1*10 allele ($n = 4$ each) was higher among patients with hypoparathyroidism who were positive for the CaSRAB as compared with those who were negative for the antibody ($n = 1$, $P = 0.02$, corrected $P = 0.36$, odds ratio = 5.2) (Table 1). However, there was no significant difference in the frequency of occurrence of any of the specific alleles of the generic HLA-DR when the 36 patients with hypoparathyroidism were segregated on the basis of the presence or absence of convulsions, cataract or intracranial calcification.

Discussion

In the present study, the incidence of intracranial calcification, convulsions, and cataract as well as the male to female ratio in the cohort of 51 patients with idiopathic hypoparathyroidism, were comparable to what has been reported earlier in the literature (26–29).

Table 2 Study variables in relation to calcium sensing receptor autoantibody status in patients with idiopathic hypoparathyroidism.

Parameters	CaSRAB +ve ($n = 25$)	CaSRAB -ve ($n = 26$)	P value
Sex (Male:Female)	15:10	14:12	0.65
Age (years)	31.9±12.4	34.3±13.5	0.50
Duration of symptoms (years)	8.3±9.3	6.2±7.7	0.38
BMI (kg/m ²)	20.7±4.1	22.0±5.1	0.68
History of convulsions	19/24 (76.0%)	13/24 (50.0%)	0.054
History of tetany	19 (76.0%)	17 (65.4%)	0.40
Cataract	12 (48.0%)	9 (34.6%)	0.33
Serum calcium (mmol/l)	1.44±0.27	1.44±0.20	0.97
Serum inorganic phosphorus (mmol/l)	2.03±0.45	2.1±0.32	0.40
Intracranial calcification	20 (80%)	19 (73.0%)	0.61
Serum intact PTH (ng/l)	10.0±9.2	9.7±7.0	0.88
Thyroid peroxidase antibody positivity	2 (8.0%)	Nil	0.99
Hypothyroidism	Nil	3 (11.5%)	0.23
Frequency of HLA-DRB1*09 allele	4/19 (21.0%)	0/17	0.065
Frequency of HLA-DRB1*10 allele	4/19 (21.0%)	1/17 (5.8%)	0.20
Frequency of HLA-DRB1*09 + DRB1*10 alleles	8/19 (42.1%)	1/17 (5.8%)	0.014

BMI, body mass index.

The present study also demonstrated the coexistence of intracranial calcification and cataract, both of which were individually associated with a longer duration of the illness. However, the duration of the illness could explain the variation in the occurrence of intracranial calcification and cataract in only 15–16% of the patients. These findings suggest a role for other factors in their aetiology.

In 1966, Blizzard *et al.* using indirect immunofluorescence had reported the presence of parathyroid autoantibodies in 33% of patients with idiopathic hypoparathyroidism (6). However, Swana *et al.* (1977) and Betterle *et al.* (1985) using similar techniques demonstrated anti-mitochondrial antibodies staining the mitochondria-rich oxyphil cells of the parathyroid gland and suggested that these could be the cause of the seropositivity observed in patients with idiopathic hypoparathyroidism, which had been reported earlier (8, 9). Using Western blot assays, Fattorossi *et al.* (1988) first described the presence of the 200 kDa and 130 kDa autoantigens in the parathyroid endothelial/epithelial cells of six patients with idiopathic hypoparathyroidism (30). Subsequently, Li *et al.* in 1996 identified the CaSR as an autoantigen and demonstrated antibodies against this receptor in 56% of 25 patients with hypoparathyroidism, 8 of whom were adults with hypoparathyroidism and coexisting thyroiditis and 17 were children with type 1 autoimmune polyglandular syndrome (14).

The inclusion of hypoparathyroidism among the autoimmune endocrinopathies associated with the autoimmune polyglandular syndrome, and the demonstration of CaSR autoantibodies in these patients indirectly suggests that similar autoimmune mechanisms might be operative in subjects with idiopathic hypoparathyroidism. Patients who have endocrinopathies linked with the autoimmune polyglandular syndrome often demonstrate a higher prevalence of organ-specific autoantibodies and exhibit autoantigens of a different type as compared with patients having the same endocrinopathies occurring in isolation without the polyglandular syndrome (31, 32). For example, Uiibo *et al.* (31) reported the presence of autoantibodies against the cytochrome P450c17, P450sc and/or P450c21 hydroxylases in 81% of their patients with Addison's disease associated with the autoimmune polyglandular syndrome while, in contrast, only 25% of the patients with isolated Addison's disease demonstrated these autoantibodies, which were found to be directed exclusively against P450c21 hydroxylase (31, 32). In the present study, the majority of patients had sporadic idiopathic hypoparathyroidism without coexisting autoimmune endocrinopathy (45/51) and 51.1% of them had serological evidence of autoantibodies against the CaSR.

In the present study, six of the 45 healthy controls (13.3%) also showed CaSR autoantibodies. Blizzard *et al.* (1966) had also reported 6% of their controls to

be positive for parathyroid autoantibodies (6). On the other hand, Li *et al.* did not detect CaSR autoantibodies in any of the 22 healthy controls studied by them (14). These variations observed in the prevalence of parathyroid autoantibodies among healthy controls may be due to differences in the number of controls studied. Interestingly, in the present study, none of the six controls who were positive for CaSR autoantibodies showed clinical or biochemical signs of hypoparathyroidism.

The CaSR protein can migrate to several different positions on SDS PAGE depending upon variations in the degree of its glycosylation or dimer formation (10, 12). Under non-reducing conditions, the 151 kDa band represents the immature, high mannose, less glycosylated form of the CaSR protein, and the band corresponding to the 168 kDa protein represents the monomeric, mature receptor which is fully glycosylated. The N-linked glycosylation of the CaSR protein is important for the normal expression of the receptor on the cell surface (11–13). The protein band analogous to a molecular weight of >250 kDa represents the dimer of the CaSR (12). Thus, in the present study, all the 25 patients had autoantibodies demonstrated against the 168 kDa CaSR autoantigen and only 4 of them had autoantibodies against the 151 kDa protein. However, while conducting the neutralisation experiment for the CaSRAb when we used parathyroid adenoma membrane preparations obtained from different patients, the commercial calcium sensing receptor antibody PA1-934 as well as the CaSRAb from the strongly positive patient's sera reacted with proteins corresponding to a CaSR molecular weight of 156 kDa and 136 kDa instead of 168 kDa and 151 kDa as observed earlier (Fig. 2B). This reactivity of the CaSR autoantibody was reduced by 90% on incubation of the serum with an excess of parathyroid membrane protein, confirming that these autoantibodies were CaSR-protein specific (Fig. 2A). However, incubation of the serum samples with the 16 amino acid synthetic analogue of the extracellular part of the CaSR (PEP-009) resulted in only a 28.4% decline in reactivity of CaSR antibody. The difference in the neutralisation of the CaSRAb observed when using parathyroid membrane preparation and PEP-009 peptide (extracellular part of the CaSR) suggests that these patients with idiopathic hypoparathyroidism may have a heterogeneous pool of autoantibodies with binding sites directed against different epitopes of CaSR (Fig. 1). Further, the CaSR autoantibody present in the serum of one of the patients with hypoparathyroidism appeared to be species specific, because although it had reacted strongly with the CaSR antigen in the human parathyroid membrane preparations, the antibody failed to react with the CaSR protein when membranes from rat liver or myocardial tissues were used (Fig. 3B).

In the present group of hypoparathyroid patients studied, we can only speculate whether the CaSR autoantibodies observed bring about the primary destruction

of the parathyroid gland or whether these autoantibodies mark the end stage of a disease process which originally begins with damage to the parathyroid gland mediated by the Th1 lymphocytes with consequent activation of the local Th2 cells, leading to the gradual development of autoantibodies, a pattern of progression which may take place in predisposed individuals with specific class II haplotypes. Brandi *et al.* have demonstrated antibody-dependent cytotoxicity in cultured bovine parathyroid cells when exposed to serum from patients with autoimmune hypoparathyroidism and type 1 APS (3). Besides, Wortsman *et al.* have demonstrated T lymphocyte activation with increased natural killer cell activity but an absence of B-cell activation in patients with adult onset idiopathic hypoparathyroidism (2). Weak immunohistochemical reactivity for both MHC class I and class II antigens has been demonstrated in dispersed normal human parathyroid cells (33). While we could demonstrate HLA-DR associations in the whole cluster of patients with idiopathic hypoparathyroidism, the CaSRAb detected in the sera of these patients did not correlate with either the clinical manifestations or the biochemical abnormalities of the disease. In diseases where autoantibodies have a primary role in pathogenesis, the clinical and biochemical severity of the illness correlates with the degree of seropositivity, for example in Graves' disease and myasthenia gravis (34, 35). Thus, among the patients in the present study the absence of any correlation of the CaSRAb with the clinical or biochemical manifestations of the disease may either represent an epiphenomenon due to immune activation or might involve antibody-dependent cell-mediated cytotoxicity.

Alternatively, the CaSRAb might lead to a decline in the PTH release essentially by acting as an agonist for binding to the CaSR that is naturally expressed on the parathyroid cell membranes. However, such a phenomenon by itself would not bring about the parathyroid gland atrophy and lymphocytic infiltration that have been observed and reported at autopsy in a few patients with idiopathic hypoparathyroidism (4). While the above facts argue against a primary role and any functional significance of the CaSRAb among patients with idiopathic hypoparathyroidism, there is a recent report of CaSR blocking autoantibodies described in patients with a syndrome resembling familial hypocalcaemic hypercalcaemia (36). However, in order to reveal the pathogenetic mechanism(s) at work, further studies need to be done *in vitro* to examine the possible role of the CaSR autoantibody in altering the normal functioning of the G-protein-mediated intracellular signalling pathways used by this receptor, as well as an analysis of the Th1 and Th2 cytokine profiles among patients with idiopathic hypoparathyroidism. In the present study, the hypoparathyroidism observed in 51% of patients in whom serological evidence of CaSRAb could not be demonstrated would still be referred to as 'idiopathic hypoparathyroidism'. In this

group, the existence of autoimmunity to antigens other than the CaSR or disappearance of the CaSRAb positivity are possible alternatives. Moreover, in the present cohort of patients, we also cannot rule out the theoretical possibility of the existence of activating mutations of the CaSR gene or altered cleavage of the PTH signal peptide due to a mutation in the prepro-PTH gene – both known causes of hypoparathyroidism. However, genetic mutations involving the CaSR and prepro-PTH gene have been reported among patients with familial isolated hypoparathyroidism and not in subjects with the sporadic form of hypoparathyroidism of the type reported in the present study (37, 38).

Multiple target gland involvement is frequent among patients with autoimmune endocrinopathies. In India, among patients with type 1 diabetes mellitus with no clinical and biochemical evidence of hypothyroidism, the prevalence of concurrent thyroid autoimmunity is 26% (39). In the present study, while we could document serological evidence of CaSRAb in half of the patients with hypoparathyroidism, the observed frequency of coexisting thyroid autoimmunity was only 9.8% which was not significantly different from that observed among the controls. This would suggest that patients with idiopathic hypoparathyroidism develop autoimmunity to the thyroid gland only infrequently.

The autoimmune nature of hypoparathyroidism warrants a study of its HLA associations. However, there is no data on HLA-DR associations among patients with idiopathic isolated hypoparathyroidism. In the present study the occurrence of the HLA-DRB1*01 or DRB1*09 alleles was significantly higher among the hypoparathyroid patients, with a relative risk of 7.8. Allelic variations were also demonstrated in relation to their CaSRAb status, with the frequency of HLA-DRB1*09 or DRB1*10 being higher in the CaSRAb-positive patients with an odds ratio of 5.2. However, the latter did not attain statistical significance. No similar association in allelic variation was observed when the patients were stratified on the basis of presence or absence of complications such as intracranial calcification or cataract. Further detailed studies involving associations with HLA class I and class II-DQB1, DQA1 and DPB1 alleles in the present group of patients might reveal such associations.

To conclude, the present study documents serological evidence of autoimmunity against a 168 kDa CaSR protein in almost half (49%) of a group of patients with sporadic idiopathic hypoparathyroidism who were investigated. The antibodies were demonstrable even up to six years after the onset of the disease. While CaSRAb seropositivity and HLA-DR associations support the autoimmune nature of the disease, the primary role of the CaSRAb in the pathogenesis of the idiopathic hypoparathyroidism needs to be further examined in view of the fact that the CaSRAb positivity did not segregate patients with regard to the frequency with which they developed convulsions, cataract, intracranial

ganglia calcification, abnormal phosphorus/calcium ratio or any other clinical manifestations of the disease studied.

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