ORIGINAL ARTICLE

Autoimmune Polyendocrine Syndrome Type 1 and NALP5, a Parathyroid Autoantigen

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ABSTRACT

BACKGROUND

Autoimmune polyendocrine syndrome type 1 (APS-1) is a multiorgan autoimmune disorder caused by mutations in *AIRE*, the autoimmune regulator gene. Though recent studies concerning *AIRE* deficiency have begun to elucidate the molecular pathogenesis of organ-specific autoimmunity in patients with APS-1, the autoantigen responsible for hypoparathyroidism, a hallmark of APS-1 and its most common autoimmune endocrinopathy, has not yet been identified.

METHODS

We performed immunoscreening of a human parathyroid complementary DNA library, using serum samples from patients with APS-1 and hypoparathyroidism, to identify patients with reactivity to the NACHT leucine-rich-repeat protein 5 (NALP5). Subsequently, serum samples from 87 patients with APS-1 and 293 controls, including patients with other autoimmune disorders, were used to determine the frequency and specificity of autoantibodies against NALP5. In addition, the expression of NALP5 was investigated in various tissues.

RESULTS

NALP5-specific autoantibodies were detected in 49% of the patients with APS-1 and hypoparathyroidism but were absent in all patients with APS-1 but without hypoparathyroidism, in all patients with other autoimmune endocrine disorders, and in all healthy controls. NALP5 was predominantly expressed in the cytoplasm of parathyroid chief cells.

CONCLUSIONS

NALP5 appears to be a tissue-specific autoantigen involved in hypoparathyroidism in patients with APS-1. Autoantibodies against NALP5 appear to be highly specific and may be diagnostic for this prominent component of APS-1.

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Autoimmune polyendocrine syndrome type 1 (APS-1) (Online Mendelian Inheritance in Man number 240300) is a rare autosomal recessive disorder that develops in early childhood and results in tissue-specific multiorgan autoimmunity, leading to the hypofunction of multiple glands.[1] Endocrine organs such as the adrenal cortex, ovaries, and parathyroid glands are typically affected, resulting in a variety of clinical diseases, including hypocortisolism, hypoaldosteronism, delayed puberty, premature ovarian failure, and hypoparathyroidism with life-threatening hypocalcemia.[2] APS-1 is clinically defined as the presence of at least two components of the classic triad of hypoparathyroidism, adrenal insufficiency, and mucocutaneous candidiasis.

APS-1 is caused by mutations in the autoimmune regulator gene (*AIRE*),[3] which encodes a 54-kD protein expressed in stromal cells of primary and secondary lymphoid tissues, including thymic medullary epithelial cells.[4] *AIRE* regulates the transcription of tissue-restricted antigens and has hence been linked to both central and peripheral tolerance.[4-8] Although the discovery of *AIRE* and its importance in APS-1 has provided new insights into the mechanisms of organ-specific immunologic tolerance, the molecular pathophysiology of APS-1 remains incompletely understood.[9]

Patients with APS-1 have autoantibodies directed against tissue-specific, key enzymes expressed in target organs affected by the disease.[10-12] The presence of these autoantibodies is generally predictive of the development of organ failure. This is best exemplified in patients with adrenal insufficiency, in whom autoantibodies are reactive to 21-hydroxylase, a key enzyme in glucocorticoid synthesis.[13-15]

Hypoparathyroidism is a hallmark of APS-1 and affects more than 80% of patients with the syndrome.[2,16] Candidate autoantigens previously reported to be linked to hypoparathyroidism, including the almost ubiquitously expressed calcium-sensing receptor, have not been confirmed as relevant autoantigens.[16-20] Thus, the identification of a parathyroid-specific autoantigen is important for improved serologic diagnosis of the disease and would provide better understanding of the molecular mechanisms underlying APS-1.

To identify a parathyroid-specific antigen, we immunoscreened a complementary DNA (cDNA) expression library derived from human parathyroid glands, using serum samples from patients with APS-1. With this approach, we identified NALP5, the NACHT leucine-rich-repeat protein 5 that also contains a pyrin domain, as an important parathyroid autoantigen in APS-1.

METHODS

ETHICAL APPROVAL

All patients and controls were included in the study only after we had obtained informed written consent. The study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee at Uppsala University.

CONSTRUCTION AND SCREENING OF THE cDNA LIBRARY

Messenger RNA (mRNA) was isolated from normal human parathyroid tissue removed during thyroid surgery. A cDNA expression library was constructed by means of the ZAP Express vector system (Stratagene) and was immunoscreened with the use of serum samples from patients with APS-1 and hypoparathyroidism. Positive clones were isolated and sequenced.

CHARACTERISTICS OF THE PATIENTS AND COLLECTION OF SERUM SAMPLES

Serum samples were obtained and analyzed from 11 Swedish, 18 Norwegian, and 58 Finnish patients with APS-1 who were members of more than 50 independent kindreds. Control serum samples from patients with isolated hypoparathyroidism (20 patients), Graves' disease (20), adrenal insufficiency (20), type 1 diabetes (20), or Sjögren's syndrome (20) and from 193 healthy blood donors were included in the analysis. The diagnosis of APS-1 was based on the presence of at least two of the three major clinical manifestations: hypoparathyroidism, adrenal insufficiency, and mucocutaneous candidiasis. The majority of the patients with APS-1 (83 of 87) were also found to have typical mutations in the *AIRE* gene.

The diagnostic criterion for mucocutaneous candidiasis was candidal infection of the oral mucosa, skin, or nails for more than 3 months. For hypoparathyroidism, the diagnostic criteria were a subnormal plasma calcium concentration (<2.15 mmol per liter) and a supranormal plasma phosphate concentration, together with a low-to-normal or low parathyroid hormone concentration and normal renal function; and for adrenal insufficiency, a subnormal serum cortisol concen-

tration, together with an elevated plasma corticotropin concentration, or a serum cortisol concentration of less than 550 nmol per liter at 30 or 60 minutes during a corticotropin stimulation test. The majority of the patients who received a diagnosis of adrenal insufficiency also had autoantibodies against 21-hydroxylase.

All the controls had normocalcemia. Tests for autoantibodies against the calcium-sensing receptor with the use of previously described methods[16] were negative in serum samples from all the patients with APS-1 in our study.

GENERATION OF ^{35}S-RADIOLABELED NALP5 AND AUTOANTIBODY ASSAY

A full-length cDNA clone for human NALP5 (catalog no. SC306608, Origene Technologies) was used for in vitro transcription and translation and labeling with ^{35}S-methionine using the TNT system (Promega) according to the manufacturer's protocol. The ^{35}S-radiolabeled recombinant NALP5 was directly immunoprecipitated with serum samples from patients or controls in 96-well filtration plates (Millipore). Serum samples from each patient or control were analyzed in a double-blind manner in duplicate, and 20,000 counts per minute (cpm) of ^{35}S-NALP5 was used for immunoprecipitation in each well. The radioactivity of the immunoprecipitated material was evaluated with the use of a liquid scintillation counter (Wallac 1450 MicroBeta, PerkinElmer). The serum sample from a patient with APS-1 that was used in the immunoscreening for NALP5 was used as the positive standard, and a serum sample from a healthy blood donor was used as the negative standard. For each analyzed serum sample, we calculated a NALP5 autoantibody index (defined as [cpm in the unknown sample − cpm in the negative standard] ÷ [cpm in the positive standard − cpm in the negative standard] × 100). The upper limit of the normal range was defined as the mean of the values obtained for the 193 healthy blood donors plus 3 SD. The intraassay and interassay coefficients of variation were 8.5% and 12.0%, respectively.

SEQUENTIAL IMMUNOPRECIPITATION

To confirm the specificity of the NALP5 autoantibodies in serum samples from patients, sequential immunoprecipitation was performed. First, recombinant ^{35}S-labeled NALP5 protein (150,000 cpm) was precipitated with the use of 2.5 μl of serum from a patient or control. Antibody–antigen complexes were captured on protein-A Sepharose beads and were subsequently removed by centrifugation. The remaining supernatant was then subjected to a second immunoprecipitation step involving NALP5-specific rabbit antiserum. The final immunoprecipitate was analyzed with the use of sodium dodecyl sulfate–polyacrylamide-gel electrophoresis followed by autoradiography.

ANALYSIS OF cDNA EXPRESSION

Normalized cDNA from a human multiple-tissue panel (BD Biosciences) and from human parathyroid gland were used as templates for a quantitative polymerase-chain-reaction (PCR) assay. Details on the primers and conditions used are provided in the Supplementary Appendix (available with the full text of this article at www.nejm.org). Samples were analyzed in triplicate.

GENERATION OF ANTISERUM AGAINST NALP5 AND IMMUNOBLOT ANALYSIS

An antiserum was developed against human NALP5 by immunizing rabbits with a keyhole limpet hemocyanin (KLH)–conjugated peptide (amino acids 897 through 910: Cys–Lys–Ser–Leu–Ser–Leu–Ala–Gly–Asn–Lys–Val–Thr–Asp–Gln–Gly). This sequence has a high homology to bovine NALP5. The antiserum obtained was affinity-purified on a peptide column. The specificity of the serum was verified by means of immunoblotting with human and bovine parathyroid cells in the absence or presence of the KLH peptide.

IMMUNOHISTOCHEMICAL ANALYSIS

Methods of immunohistochemical analysis are described in the Supplementary Appendix.

IMMUNOFLUORESCENCE AND LASER-SCANNING CONFOCAL-MICROSCOPICAL ANALYSIS

Cryosections (6 μm in thickness) of bovine parathyroid tissue were air-dried, blocked with normal goat serum, and incubated with serum samples (1:500 dilution) from patients with APS-1 who had NALP5 reactivity. Serum samples from healthy blood donors and from patients with APS-1 but without NALP5 autoantibodies were used as a negative standard, and affinity-purified rabbit anti-NALP5 antiserum was used as a positive

standard. The slides were incubated with fluorescein isothiocyanate–conjugated secondary antibodies (dilution, 1:200) for 30 minutes and were then analyzed on a confocal microscope (LSM 510, Zeiss). To verify the specificity of the serum samples from patients with APS-1 for NALP5, we performed absorption studies using serum samples from three patients with NALP5-specific autoantibodies and a positive staining pattern on immunofluorescence. Serum samples from these patients were diluted (1:500) to a final volume of 500 μl and incubated overnight with either 15 μl of phosphate-buffered saline (as a negative control), 15 μl of TNT-derived recombinant ^{35}S-radiolabeled NALP5 (approximately 450,000 cpm), 15 μl of TNT-derived recombinant ^{35}S-radiolabeled NALP3 (approximately 470,000 cpm), or 15 μl of TNT-derived recombinant luciferase (approximately 1 million cpm). After absorption overnight, the serum samples were tested by means of immunofluorescence on bovine parathyroid tissue, as described above.

STATISTICAL ANALYSIS

Fisher's exact test was used to determine the association of reactivity to NALP5 with the major clinical manifestations of APS-1 in patients with the syndrome.

RESULTS

SCREENING FOR NALP5-SPECIFIC AUTOANTIBODIES

To identify tissue-specific autoantibodies, immunoscreening of a parathyroid cDNA expression library was performed with the use of serum samples from a patient with APS-1 who had severe hypoparathyroidism. Two independent clones, both encoding exons 5 through 14 of the human NALP5 gene (GenBank accession no. AY154460), were identified.

We verified the frequency and specificity of autoantibodies to NALP5 by means of immunoprecipitation, using ^{35}S-methionine–labeled human NALP5 protein generated by in vitro transcription and translation. NALP5-specific autoantibodies were detected in serum samples from 36 of the 87 patients with APS-1 (41%). These autoantibodies were highly specific for hypoparathyroidism associated with APS-1, since no reactivity was found in patients with APS-1 but without hypoparathyroidism, in patients with isolated hypoparathyroidism, in patients with other autoimmune endocrinopathies, or in healthy controls (Table 1 and Fig. 1A).

To confirm the specificity of NALP5-specific autoantibodies in serum samples from the patients, sequential immunoprecipitation with ^{35}S-labeled

Table 1. Associations between Clinical Manifestations of Autoimmune Polyendocrine Syndrome Type 1 (APS-1) and the Presence of NACHT Leucine-Rich-Repeat Protein 5 (NALP5) Autoantibodies.*

Manifestation	APS-1	APS-1 and NALP5 Autoantibodies		P Value
		With Manifestation	Without Manifestation	
		number/total number (percent)		
Hypoparathyroidism	73/87 (84)	36/73 (49)	0/14	<0.001
Hypogonadism	28/87 (32)	19/28 (68)	17/59 (29)	<0.001
Adrenal insufficiency	69/87 (79)	29/69 (42)	7/18 (39)	0.81
Type 1 diabetes mellitus	11/87 (13)	2/11 (18)	34/76 (45)	0.10
Vitiligo	17/87 (20)	7/17 (41)	29/70 (41)	0.99
Alopecia	30/87 (34)	11/30 (37)	25/57 (44)	0.52
Hepatitis	15/87 (17)	8/15 (53)	28/72 (39)	0.31
Malabsorption	22/87 (25)	10/22 (45)	26/65 (40)	0.66
Pernicious anemia	14/87 (16)	5/14 (36)	31/73 (42)	0.64
Candidiasis	83/87 (95)	34/83 (41)	2/4 (50)	0.72

* Fisher's two-tailed exact test was used to compare the data between the two groups of patients with autoantibodies against NALP5 and the P values reported. Although a significant correlation was found for hypogonadism, NALP5 autoantibodies are not specific for this manifestation, since 29% of the patients without hypogonadism also have autoantibodies against NALP5.

human NALP5 protein was performed. We used serum samples from patients with APS-1 and NALP5 autoantibodies that were detected on radioimmunoassay and on immunostaining of parathyroid tissue (see below), samples from patients without NALP5 autoantibodies, and samples from healthy controls. The second immunoprecipitation was performed with the use of a rabbit antiserum against NALP5. The ^{35}S-radiolabeled recombinant NALP5 protein was depleted only with the serum samples from patients with APS-1 and hypoparathyroidism, showing the specificity of the patients' autoantibodies for NALP5 (Fig. 1B, lanes 3 through 5 vs. lanes 6 through 8).

DISTRIBUTION OF NALP5 mRNA AMONG TISSUES

We also examined the expression of NALP5 in various organs, using quantitative PCR methods. NALP5 mRNA was predominantly expressed in parathyroid tissue (Fig. 2A). Moreover, NALP5 transcripts were also, albeit to a lesser extent, detected in ovaries and in a few additional types of tissue in which other members of the NALP-protein family with high homology to NALP5 were also detected (Fig. 2B, and Table 1 in the Supplementary Appendix).

PARATHYROID IMMUNOSTAINING

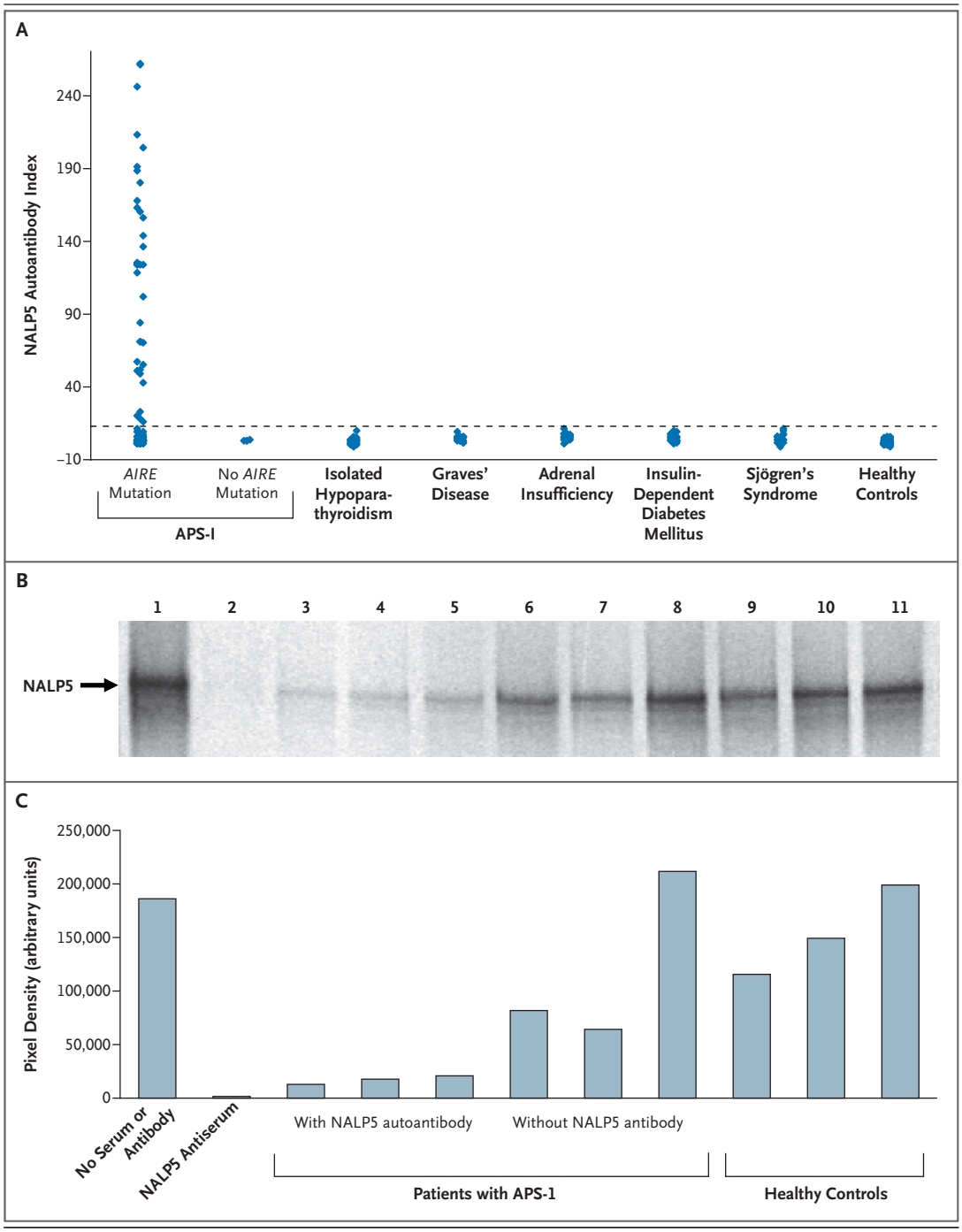
Human parathyroid glands were subjected to immunostaining with serum samples from patients with APS-1 and hypoparathyroidism (Fig. 3). These serum samples (Fig. 3B), as well as the rabbit antiserum (Fig. 3D), specifically identified parathyroid chief cells, whereas oxyphilic cells were not recognized. Identical immunofluorescence results were obtained with the use of bovine tissue (Fig. 3E through 3J). The observed reactivity was specific, since there was no staining of parathyroid tissue by serum samples from patients with APS-1 who did not have NALP5 autoantibodies or by serum samples from healthy controls. Laser-scanning confocal microscopy revealed that the subcellular localization of NALP5 in bovine parathyroid tissue was within the cytoplasm (Fig. 3J). The specificity of NALP5-autoantibody immunostaining of parathyroid-tissue specimens was further confirmed by absorption studies, in which preabsorption of the serum samples with recombinant NALP5 blocked the parathyroid staining (Fig. 4B). In contrast, preabsorption of the serum samples with equal amounts of recombinant NALP3 or luciferase did not block specific staining of parathyroid tissue (Fig. 4C and 4D).

Figure 1 (facing page). Presence of Autoantibodies against NALP5 in Patients with Autoimmune Polyendocrine Syndrome Type 1 (APS-1).

Panel A shows the results of the comparison of NALP5 autoantibody titers in serum samples from 87 patients with APS-1 (83 of whom had the *AIRE* mutation and 4 of whom did not), 100 patients with other autoimmune disorders (5 disorders and 20 patients with each), and 193 healthy blood donors. The dashed line indicates the upper limit of the normal range, defined as the mean of the values obtained for the healthy blood donors plus 3 SD. Panel B shows the results of confirmation of the specificity of autoantibodies by means of sequential immunoprecipitation of ^{35}S-methionine–radiolabeled human parathyroid NALP5. For the first immunoprecipitation step, we used no antibody or serum (positive standard, lane 1), NALP5 antiserum (negative standard, lane 2), serum samples from each of three patients with reactivity to NALP5 (lanes 3, 4, and 5), serum samples from each of three patients without reactivity to NALP5 (lanes 6, 7, and 8), and serum samples from healthy controls (lanes 9, 10, and 11). Panel C shows the results of autoradiography of the blots shown in Panel B by means of semiquantitative measurement of pixel density with ImageQuant software.

DISCUSSION

Hypoparathyroidism is the most prevalent endocrinopathy in patients with APS-1 and, in some, is the sole endocrine disorder.[16] Despite its clinical importance, a parathyroid-specific autoantigen has not yet been identified.[19] Our studies indicate that NALP5, which is selectively expressed in the cytoplasm of chief cells in the parathyroid glands, may act as an autoantigen in patients with APS-1 in whom hypoparathyroidism develops, giving rise to NALP5 autoantibodies. Moreover, NALP5 appears to be a common autoantigen for both APS-1 and its corresponding animal model, the *AIRE*-deficient mouse (unpublished data). This finding will allow for further studies in the mouse model to elucidate the exact molecular mechanisms leading to loss of tolerance against NALP5 in the absence of AIRE. Although the exact role of NALP5 autoantibodies, as well as other autoantibodies directed against intracellular autoantigens such as glutamate decarboxylase, remains unknown, these autoantibodies may serve as important markers for active cell-mediated immune responses. Our findings suggest that the detec-



tion of NALP5-specific antibodies has a specificity of 100% and a sensitivity of 49% for the diagnosis of hypoparathyroidism.

Although the assay we used detected NALP5 autoantibodies in 49% of patients, the failure to detect NALP5 autoantibodies in the remaining 51% of the patients with hypoparathyroidism has several possible explanations. First, since hypoparathyroidism is usually the first endocrine manifestation of APS-1, there may be a decrease in the titer of NALP5 autoantibodies over time in patients with long-standing disease.[21] Second, differences in tertiary structure between native NALP5 and the recombinant ^{35}S-labeled NALP5

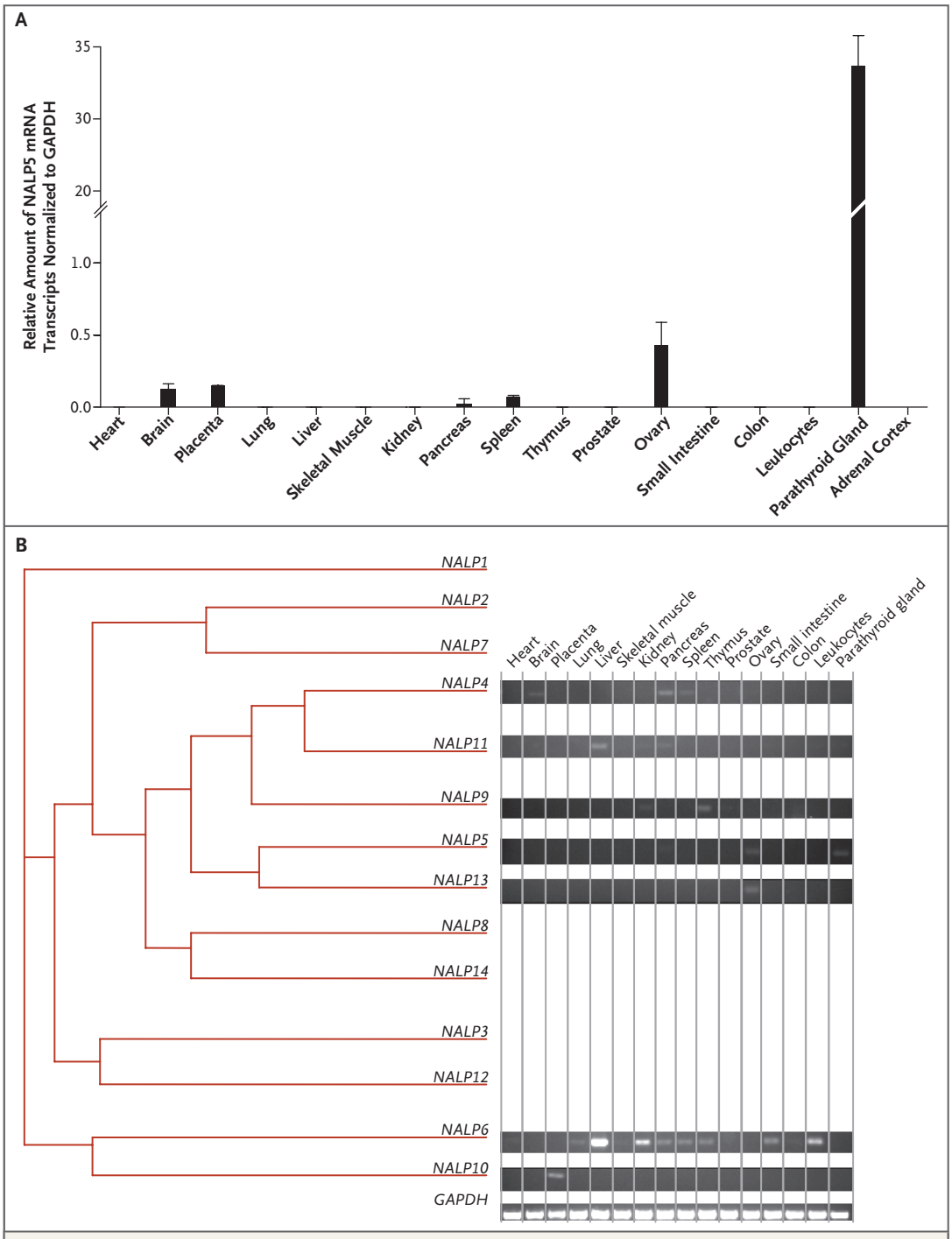


Figure 2. Analysis of the Expression of Messenger RNA (mRNA) of NALP5 and Its Homologues.

Panel A shows the expression of NALP5 mRNA in adult human tissues as measured by quantitative PCR assay. Note the noncontinuous y axis and its variable intervals. T bars are standard deviations. Panel B shows a phylogenetic tree (designed with ClustalW software) on the left-hand side indicating homologies among mRNA in the NALP protein family and mRNA expression in a human multiple-tissue panel on the right-hand side. Each of the selected proteins in the NALP protein family was detected by means of a 38-cycle conventional PCR assay involving the complementary DNA (cDNA) from the tissue panel. Note the band corresponding to *NALP5* for the parathyroid-gland specimen. These experiments were performed to exclude the expression of NALP5 homologues in the parathyroid glands. Samples of cDNA from the adrenal cortex were not yet available when these experiments were performed. GAPDH denotes glyceraldehyde-3-phosphate dehydrogenase.

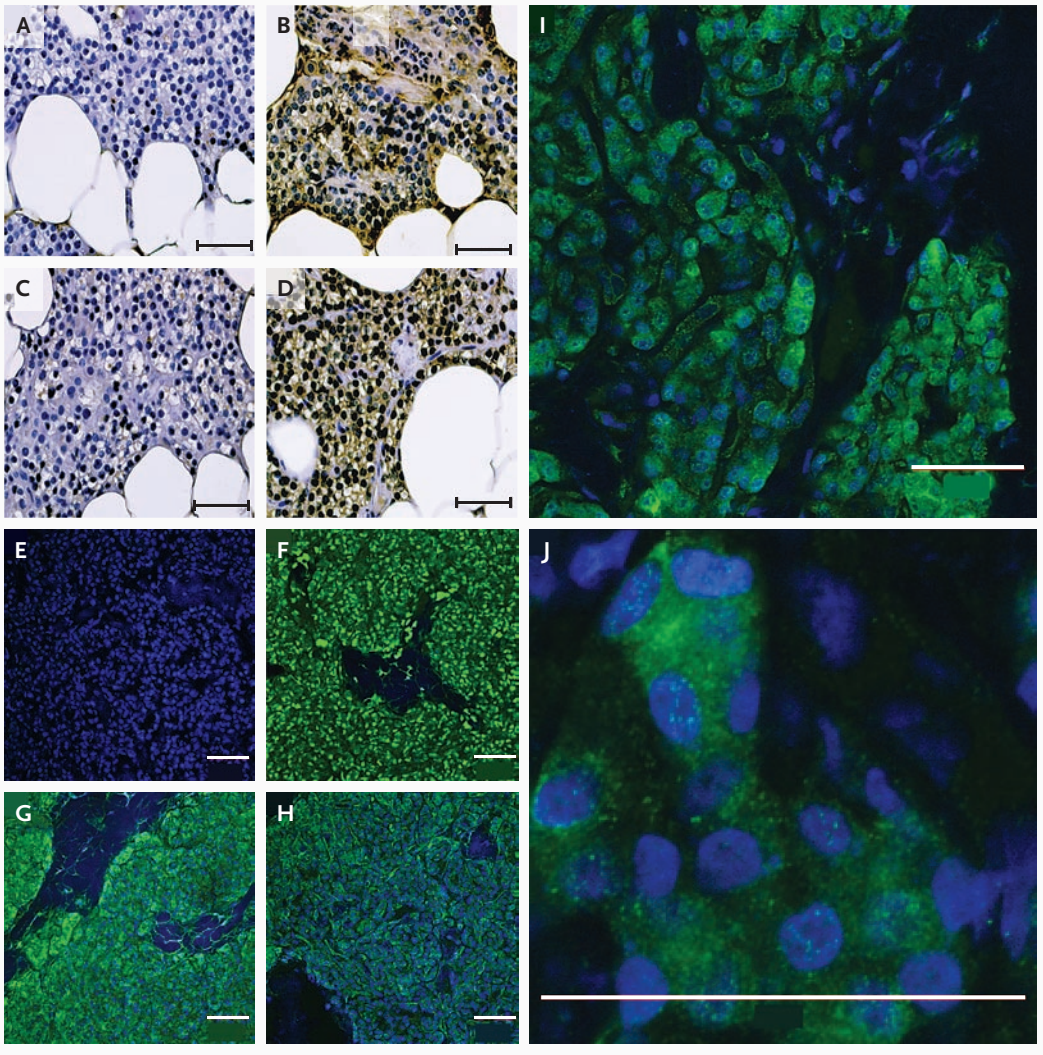


Figure 3. Immunostaining on Sections from Human and Bovine Parathyroid Glands.

Results of immunohistochemical analysis of sections from a human parathyroid gland are shown in Panels A through D; immunofluorescence of cryosections of bovine parathyroid gland is shown in Panels E through J. Background staining is shown in Panels A and E, by omission of primary serum samples. Staining patterns with serum samples are also shown: samples from a patient with autoimmune polyendocrine syndrome type 1 (APS-1) and hypoparathyroidism (Panels B and F), samples from a patient with APS-1 without hypoparathyroidism (Panels C and G), samples from a healthy control (Panel H), and samples of rabbit anti–NACHT leucine-rich-repeat protein 5 (NALP5) antiserum (Panels D, I, and J). Primary antibodies were developed with the use of a fluorescein isothiocyanate–labeled secondary antibody (green); a nuclear counterstain including 4′,6-diamidino-2-phenylindole (blue) was also used. Scale bars represent 50 μm.

used in the assay might have affected the binding of autoantibodies, leading to suboptimal sensitivity of the autoantibody assay. Although our results suggest that NALP5 is the major parathyroid autoantigen in patients with APS-1, the existence of other autoantigens cannot be ruled out.

Autoimmune ovarian disease is diagnosed in more than half of female patients with APS-1 who present with either premature ovarian failure or delayed puberty.[2] Since NALP5 is expressed in ovaries, it may also act as an ovarian autoantigen or an ovarian source of autoantigen in women that is important for the development of hypoparathyroidism. We have observed a correlation between the presence of NALP5-specific autoantibodies and autoimmune ovarian insufficiency

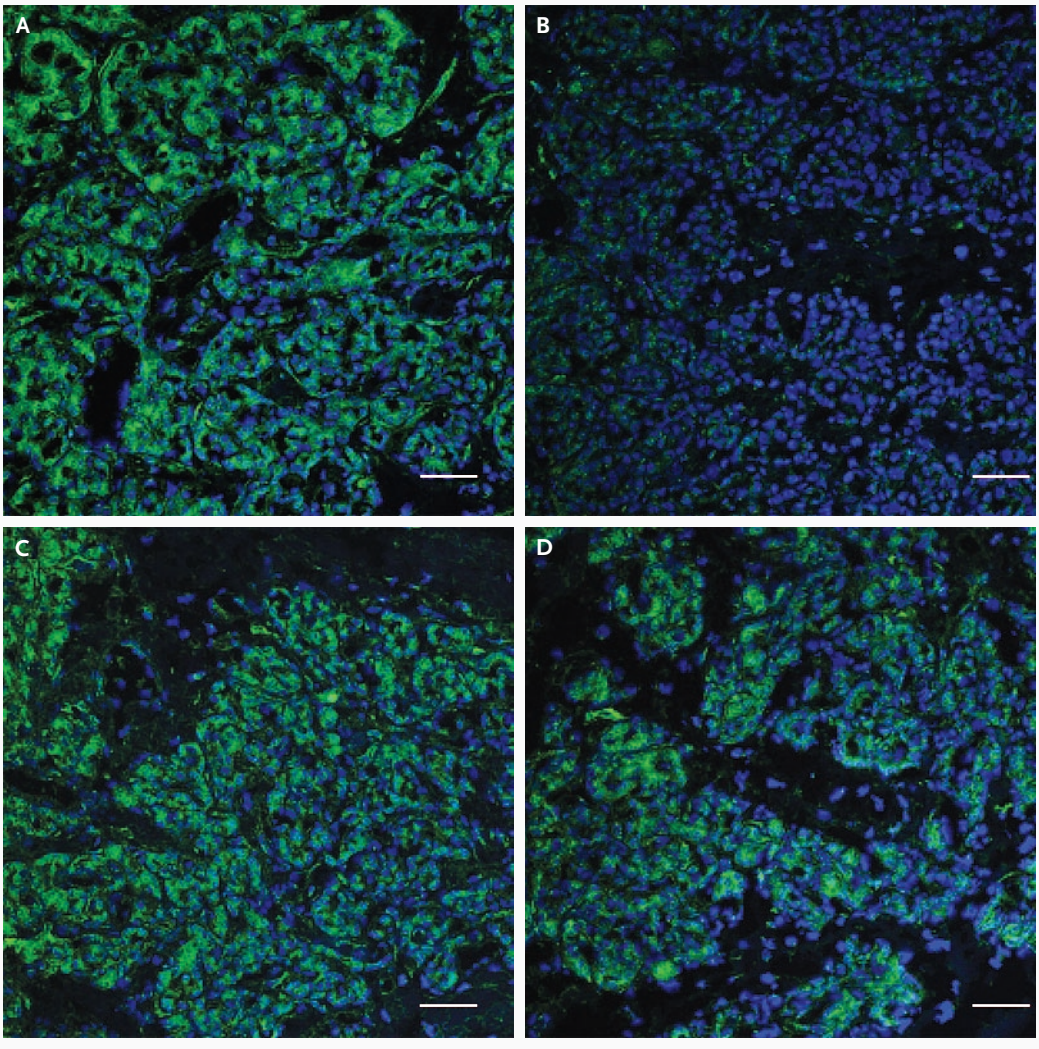


Figure 4. Results of Absorption Studies to Confirm the Specificity of Autoantibodies for NACHT Leucine-Rich-Repeat Protein (NALP) 5 in Patients with Autoimmune Polyendocrine Syndrome Type 1 (APS-1).

Immunofluorescence was performed on cryosections of bovine parathyroid gland with the use of serum from a patient with APS-1 and NALP5 autoantibodies. The serum was diluted (1:500) and preincubated with either no protein (Panel A) or an equal amount of in vitro transcribed and translated NALP5 labeled with ^{35}S-methionine (Panel B), NALP3 (Panel C), or luciferase (Panel D). Scale bars represent 50 μm.

(Table 1), supporting such a conclusion. However, all our patients who had NALP5 autoantibodies and hypogonadism also had hypoparathyroidism, illustrating that NALP5-specific autoantibodies are neither specific nor independent markers for hypogonadism. The autoantigen identified in mice thymectomized at day 3 of life, commonly used as an animal model for autoimmune ovarian disease,[22-24] is the ooplasm-specific Mater (maternal antigen that embryos require) protein[25,26] — the mouse orthologue of NALP5.

It has been noted that hypoparathyroidism is the only disease component in patients with APS-1 that has a distinct difference in prevalence between the sexes, occurring in 98% of female patients but in only 71% of male patients.[19] This difference is in keeping with the differential expression patterns of NALP5. Although NALP5 expression is restricted to the parathyroid glands in men, it is found in both the parathyroid glands and the ovaries in women. This expression pattern may therefore increase the exposure of this

autoantigen in women and may explain the observed sex difference in the prevalence of hypoparathyroidism among patients with APS-1.[19]

The subcellular localization of NALP5, the presence of a C-terminal leucine-rich repeat, and the presence of the ATP–GTP binding domain all suggest that NALP5 has a role in cell signaling. The caterpillar family of proteins, to which NALP5 belongs, shows an ability of pattern recognition of larger microbial molecules as well as smaller substances.[27] Indeed, NALP5 expression is up-regulated in parathyroid cells subjected to increasing calcium concentrations (unpublished data). Hence, NALP5 may be involved in the process of calcium sensing or homeostasis in parathyroid chief cells. Such a putative function of NALP5 would be in keeping with its alleged role as a maternal-effect gene, as reported during early mouse development.[26] Beyond the two-cell stage of embryonic development, single-cell autonomous or localized signaling shifts to global intercellular calcium signaling, which parallels the increase in the number of embryonic cells, the decrease in cell size, and the events of cellular rearrangement.[28]

The identification of NALP5 as a target of the autoimmune response in patients with APS-1 could provide unique insights into the molecular mechanisms that initiate the autoimmune disorder that eventually leads to the functional loss of the parathyroid gland. For example, it may be possible to detect T cells specific for NALP5 peptides in patients with APS-1, to determine the frequency and function of such cells, and to correlate their presence with the occurrence of hypoparathyroidism. Moreover, therapies directed at the reduction of the clonal expansion of NALP5-reactive T cells in an antigen-specific manner may have a protective effect in the early stages of the disease. Alternatively, long-term tolerance to NALP5 might be gained, as suggested for other autoimmune disorders,[29] by the antigen-specific induction of regulatory T cells. Indeed, CD4+CD25+ regulatory T cells are impaired in patients with APS-1 and are thus a target for immunotherapy.[30]

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