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(54) **DIFFERENTIAL DIAGNOSIS FOR SCLERODERMA**

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(57) **ABSTRACT**

Use of IFI16 protein, fragments or peptides thereof for differential diagnosis of the limited cutaneous form of scleroderma (lc-SSc) in a subject suspected of or at risk of having an autoimmune disease and the corresponding method of diagnosis and kit.

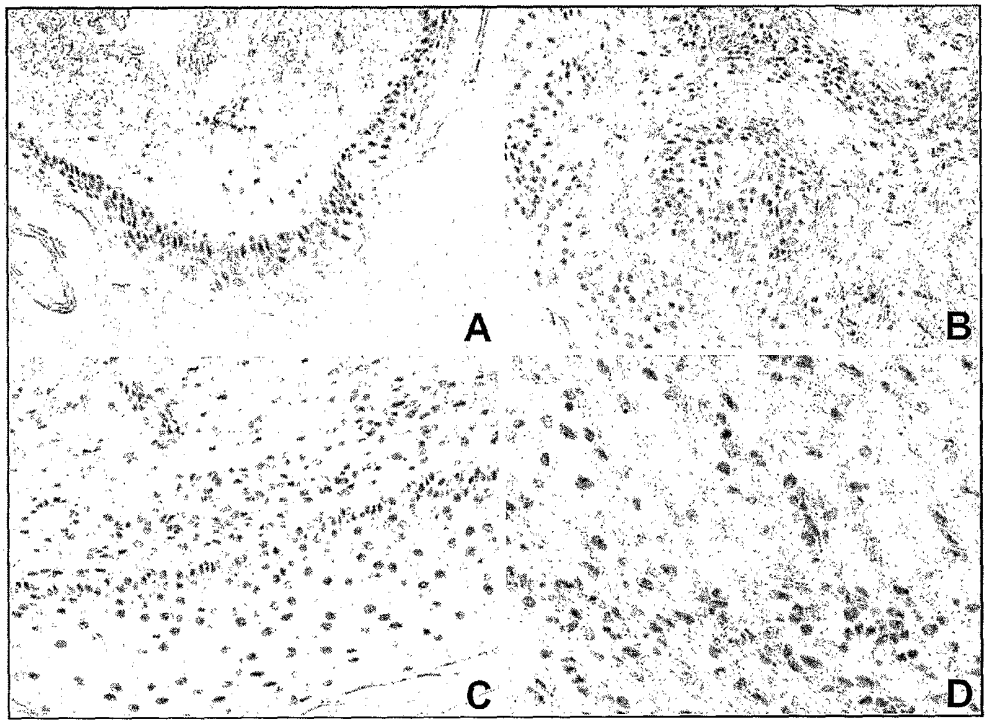

FIG. 1

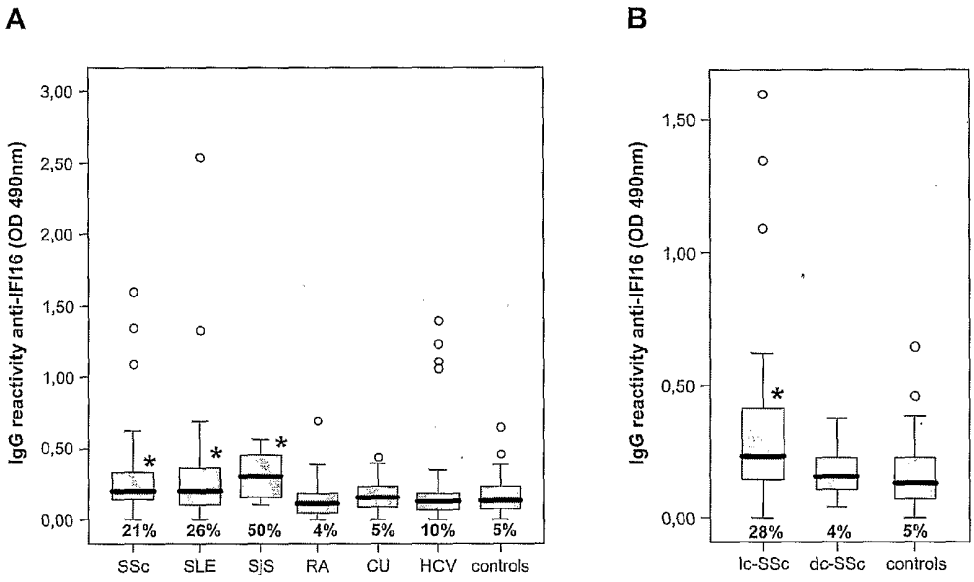
A
IgG reactivity anti-IFI16 (OD 490nm)
3,00
2,50
2,00
1,50
1,00
0,50
0,00
21%
26%
50%
4%
5%
10%
5%
SSc
SLE
SjS
RA
CU
HCV
controls
B
IgG reactivity anti-IFI16 (OD 490nm)
1,50
1,00
0,50
0,00
28%
4%
5%
lc-SSc
dc-SSc
controls

FIG. 2

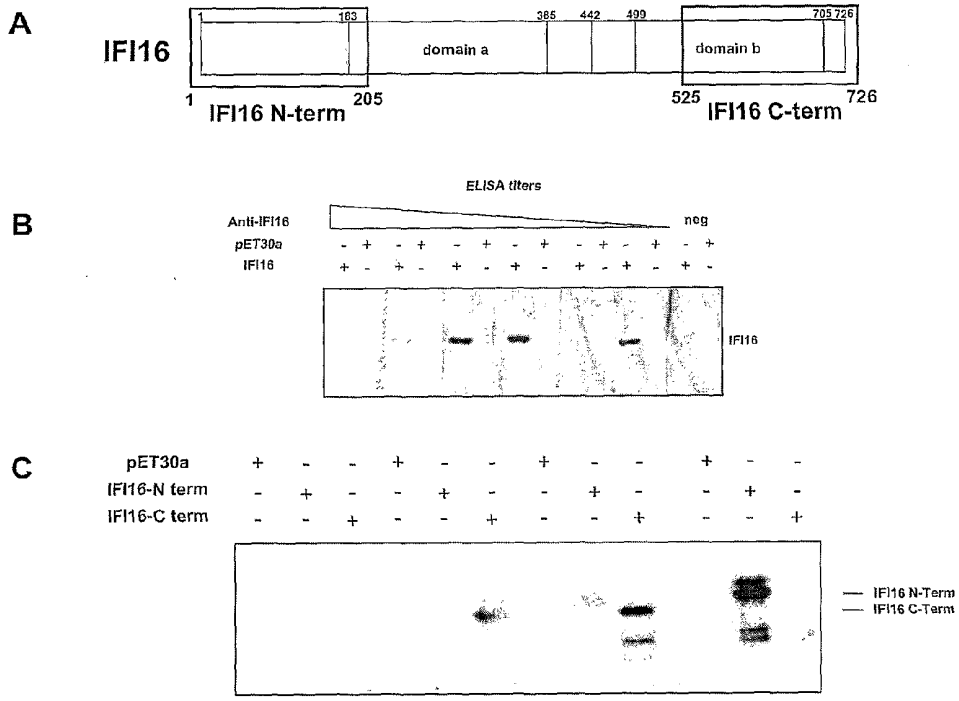
A
IFI16
1
163
domain a
385
442
499
705 726
domain b
1
205
IFI16 N-term
525
726
IFI16 C-term
B
ELISA titers
Anti-IFI16
neg
pET30a
IFI16
IFI16
C
pET30a
IFI16-N term
IFI16-C term
IFI16 N-Term
IFI16 C-Term

FIG. 3

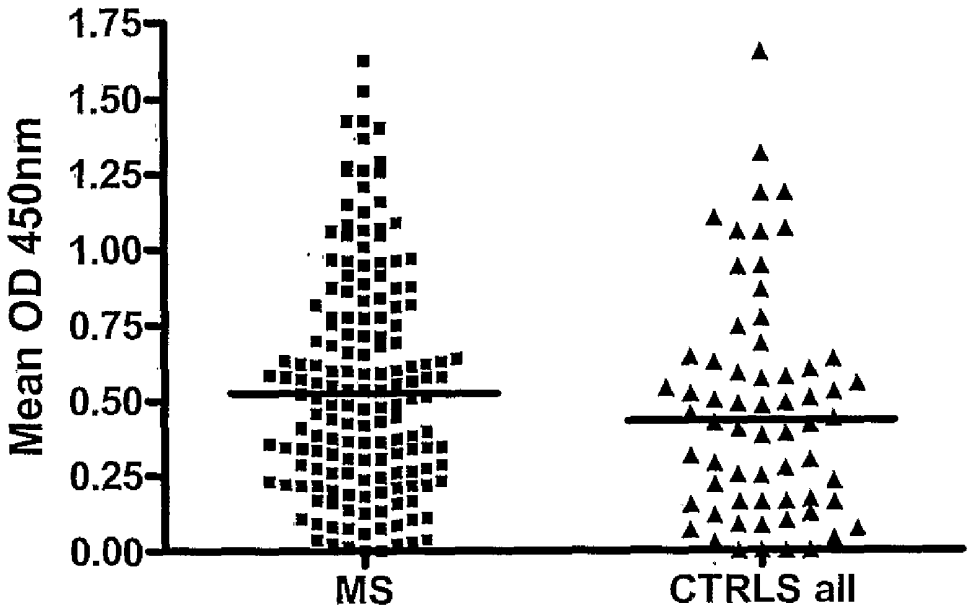

FIG. 4

DIFFERENTIAL DIAGNOSIS FOR SCLERODERMA

TECHNICAL FIELD OF THE INVENTION

[0001] The present invention concerns the presence and clinical significance of autoantibodies directed against the interferon-inducible gene IFI16 in patients with systemic sclerosis/scleroderma (SSc), systemic lupus erythematosus (SLE) and other autoimmune diseases.

BACKGROUND OF THE INVENTION

[0002] A family of interferon (IFN)-inducible genes, designated HIN200 in the human and Ifi200 in the murine species, encodes evolutionarily related human (IFI16, IFIX, MNDA, and AIM2) and murine proteins (p202, p203, p204, p205/D3) (1, 2). The IFI16 (Pubmed Accession No. NP_005522), p202, and p204 nuclear phosphoproteins participate in the inhibition of cell cycle progression, modulation of differentiation, and cell survival. Gene expression analyses in congenic mice have identified p202 as a candidate gene for lupus susceptibility (3).

[0003] In 1994 Seelig et al. (4) reported the presence of anti-IFI16 antibodies in 29% of sera obtained from systemic lupus erythematosus (SLE) patients and recently Uchida et al. (5) in up to 70% of patients suffering from both primary and secondary Sjögren's syndrome (SjS). Lower prevalence has been reported in other systemic autoimmune diseases such as rheumatoid arthritis (RA) (0-13%) and scleroderma/polymyositis overlap syndrome (3%) (4, 5). However, the studies on SjS and RA enrolled small series of patients and no data are available on the presence of these autoantibodies in another frequent systemic autoimmune disease such as scleroderma (SSc).

[0004] Moreover, IFI16 expression in target tissues of the autoimmune process (i.e. the salivary glands) was described (5). This finding raises the possibility that a local tissue expression (or even up-regulation) can be pivotal in triggering an autoimmune response against this protein. Interestingly, physiological IFI16 expression was found in vascular endothelial cells and in stratified squamous epithelia such as skin (6). Both these tissues are targets for main SLE clinical manifestations (7). The present inventors looked at IFI16 expression in the lesional skin from SLE patients in order to investigate whether an enhanced IFI16 expression might be associated with the occurrence of autoantibodies directed against it. Since an increased serum levels of anti-IFI16 antibodies was also observed in SSc patients, the present inventors extended the study to SSc skin biopsies as well.

SUMMARY OF THE INVENTION

[0005] The present invention relates to the expression of IFI16 in skin biopsy specimens from SSc and SLE patients. Levels of autoantibodies against IFI16 were determined by enzyme-linked immunosorbent assay (ELISA) in serum samples from 82 SSc and 100 SLE patients. Other autoimmune diseases (primary Sjögren's syndrome (SjS), rheumatoid arthritis (RA) and chronic urticaria (CU) were also examined.

[0006] The first aim of the present invention is to evaluate the presence and the titers of anti-IFI16 autoantibodies in a larger number of SjS, RA as well as in SSc patients and controls, and to partially characterize their antigenic specificity.

[0007] Object of the present invention is the provision of evidence that an IFN-inducible gene, IFI16, is involved in the pathophysiological mechanisms of connective tissue disorders such as SSc. More specifically, an object of the present invention is the provision of differential diagnosis methods for scleroderma, and, in particular, a novel tool in the differential diagnosis of lc-SSc (limited cutaneous form of scleroderma) from dc-SSc (diffused cutaneous form of scleroderma).

[0008] According to the present invention, said objects are achieved thanks to the solution having the characteristics referred to specifically in the ensuing claims. The claims form integral part of the technical teaching herein provided in relation to the present invention.

[0009] According to the present invention, said objects are achieved by means of the use of IFI16 protein, fragments or peptides thereof, wherein autoantibodies against IFI16 protein fragments or peptides thereof are detected. In a preferred embodiment, the diagnosis is based on the detection by an immunoassay (like for example ELISA, RIA, immunofluorescence or immunohistochemistry assay) of a higher level of autoantibodies against IFI16 protein, fragments or peptides thereof in the subject as compared to the normal level of these autoantibodies and/or to a level of these autoantibodies in patients suffering of a diffused cutaneous form of scleroderma (dc-SSc).

[0010] The differential diagnosis method according to a preferred embodiment of the present invention comprises:

[0011] a. obtaining a sample from a patient;

[0012] b. contacting the sample with an antigen comprising IFI16 protein, fragments or peptides thereof to form a complex of the autoantibody and the antigen;

[0013] c. detecting the presence of the complex autoantibody-antigen.

[0014] According to a preferred embodiment, the antigen of human, animal, synthetic or recombinant origin is bound to a solid carrier and the detection is performed using an anti-human IgG antibody, preferably conjugated to a detectable marker. The detectable marker is preferably selected from horseradish peroxidase, alkaline phosphatase, biotin or fluorescent dyes. The sample can be selected from whole blood, serum, plasma, saliva, tears, sweat, synovial fluid.

[0015] The present invention is also directed to a kit for the determination of anti-IFI16 autoantibodies in a sample preferably comprising a plate of wells having bound thereto IFI16 protein, fragments or peptides thereof and a detection reagent.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The present invention will now be described in detail in relation to some preferred embodiments by way of non-limiting examples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIG. **1**. Increased expression of IFI16 in SLE and SSc lesions compared with skin from healthy controls. Expression of IFI16 in skin from healthy control (A), SLE lesion (B) and SSc lesion (C) (Original magnification×10). D, Enlarged section of derma from Panel C showing IFI16 staining in inflammatory cells (Original magnification ×20).

[0018] FIG. **2**. A, IgG titers against human recombinant IFI16 in patients with SSc (82), SLE (100), primary SjS (20), RA (50), CU (38) and HCV infection (40) and from healthy

controls (80). Boxes show values within 25th and 75th percentiles, the horizontal bar represents the median, 80% of values are between the extremities of the vertical bars (10th-90th percentiles), and extreme values are represented by individual symbols. Differences between the groups have been evaluated by one-way ANOVA and Bonferroni Multiple Comparisons tests after logarithmic transformation. Values under the boxes represent the percentage of subjects with IgG titers above the cut off value, calculated at the 95th percentile of the control population. Statistical significance: *p<0.001 vs controls. B, IgG titers against human recombinant IFI16 in patients with lc-SSc (57), dc-SSc (25), and healthy controls (80). Graphical representation and statistical analysis as in panel A. Statistical significance: * p=0.02 vs dc-SSc and controls.

[0019] FIG. **3**. A, Schematic representation of IFI16 and a series of recombinant IFI16 fragments. The two HIN200 domains (domain a and domain b) are indicated. Recombinant peptides corresponding to IFI16 aa 1-204 (IFI16 N-term) and aa 525-726 (IFI16 C-term) are indicated by a box. B, Reactivity to full length IFI16 by immunoblotting. Recombinant IFI16 (IFI16) or control peptide (pET30a) were separated by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with 1:100 dilution of patients sera that were positive (lanes 1-12, with decreasing titers) or negative (lanes 13-14) using ELISA. C, Reactivity to IFI16 N-terminal (IFI16 N-term), C-terminal (IFI16 C-term) fragments or control peptide (pET30a). Nitrocellulose membranes with transferred recombinant proteins were incubated with patients sera that were reactive using immunoblotting to full length IFI16.

[0020] FIG. **4**. IgG titers against human recombinant IFI16 in patients with multiple sclerosis (MS, N=163) and healthy individuals (CTRL, N=64) as normal controls examined by ELISA with recombinant IFI16 protein. The horizontal bar represents the median.

[0021] The present inventors showed, for the first time, enhanced expression levels of the interferon-inducible protein IFI16 in the epidermis and in the dermal inflammatory infiltrate from both SLE and SSc lesions by immunohistochemistry. Additionally, the present inventors confirmed that anti-IFI16 autoantibody titers above the 95th percentile of the controls are significantly elevated in patients with SLE and SjS, but not in those with other autoimmune diseases compared with controls. Interestingly, the present inventors found comparable prevalence of these autoantibodies in SSc as well. Furthermore, the association of anti-IFI16 autoantibody levels in SSc patients with a range of clinical and laboratory parameters was assessed by univariate analysis. The results obtained demonstrated that anti-IFI16 autoreactivity was not associated with either disease duration or disease severity, but with the limited cutaneous form of SSc (lc-SSc).

[0022] IFI16 expression was, in fact, greatly increased and found to be ubiquitously expressed in all layers of the epidermis and in the dermal inflammatory infiltrate in the lesional skin from both SLE and SSc patients. Patients with SLE, SjS and SSc exhibited significantly (one-way ANOVA p<0.0001) higher anti-IFI16 antibody levels of the IgG isotype than normal controls (SLE p<0.002, SjS p<0.001 and SSc p<0.0005, respectively). Anti-IFI16 titers above the 95th percentile of the controls were observed in 26% of SLE, 50% of SjS and 21% of SSc respectively. By contrast, anti-IFI16 prevalence was 4% in RA, 5% in CU and 10% in HCV patients respectively.

[0023] The most striking data came from logistic regression analysis of the three serological autoimmune markers (anti-IFI16, anti-centromere and anti-topoisomerase I autoantibodies), strongly associated with the cutaneous form of SSc in the patients. This analysis showed that all three serological markers investigated were independent predictors of the cutaneous form of scleroderma, and their combination was able to explain 62% of the associated variability. Without being bound to any specific theory, the present inventors believe that IFI16 reactivity in patient with SSc is an important clue to the development of lc-SSc in anti-centromere and anti-topoisomerase I negative patients. In addition, the finding of anti-IFI16 positivity allowed the present inventors to detect lc-SSc patients among the subgroup negative for both anti-centromere and anti-topoisomerase I reactivity, indicating that determination of IFI16 reactivity in patient with SSc is a valuable tool for the differential diagnosis of lc-SSc in the double negative patients.

[0024] Prominent IFI16 expression has been seen in vascular endothelial cells and in stratified squamous epithelia such as skin. Its expression is normally restricted to the basal proliferative layer, suggesting a possible role in the control of skin homeostasis. Transduction of IFI16 into the human umbilical vein endothelial cells (HUVEC) by recombinant viruses efficiently suppressed the formation of capillary-like structures in vitro and cell-cycle progression associated with cell death (8). In addition, type I IFN released by plasmacytoid dendritic cells accumulated in cutaneous SLE lesions mediates inflammation and expression of interferon-inducible genes.

[0025] The present invention shows, then, that IFI16 is expressed to a higher level in SSc and SLE lesions in both epithelial and inflammatory cells, and autoantibody titers against it are significantly elevated in both diseases.

[0026] Thus, the disease model the present inventors propose is: i) IFI16 expression in lesional skin is enhanced by local type I IFN production or other pro-inflammatory stimuli; ii) IFI16 release, as a consequence of increased cell death, leads to the breakdown in tolerance to this self-antigen as confirmed by the generation of specific anti-IFI16 autoantibodies; iii) an additional pathogenic role of IFI16 is suggested by the observation that its endothelial expression triggers apoptosis, up-regulates the expression of genes encoding adhesion molecules (ICAM-1, E-selectin) and chemokines (IL-8, MCP-1) (S.L., unpublished data) and efficiently suppresses formation of capillary-like structures in vitro.

[0027] Finally, although the root causes of these autoimmune diseases are not yet known, with this disease model the present inventors contribute, and advance, the understanding of the cell and molecular mechanisms that impact on, and underlie, SSc and SLE. Moreover, antibody titer against IFI16 protein is an important serologic marker for the laboratory testing for the differential diagnosis of the limited cutaneous form of SSc.

[0028] Materials and Methods

[0029] Patients and Controls

[0030] 290 patients were included in this study, classified as follows: 100 SLE, 20 primary SjS, 82 SSc, RA, 38 CU and 163 MS. Sera from 80 sex and age matched healthy subjects were collected from blood banks and represented the control group. 40 patients with chronic HCV infection (kindly provided by Dr. Mario Pirisi, University of Piemonte Orientale, Novara) were also included as additional controls. SSc and

primary SjS sera were from Spedali Civili, Brescia. SLE, RA and CU sera were obtained from Istituto Auxologico Italiano, Milan.

[0031] All SSc patients (73 women and 9 men, age 21-80, mean age 57) were classified as lc-SSc or dc-SSc according to the criteria of LeRoy et al (9). Patients were regularly assessed using a published consensus core set of variables (10). Disease severity was assessed using the preliminary Medsger scale (11). The most severe involvement was considered for a global severity score. Disability index was evaluated by Health Assessment Questionnaire (HAQ) (12).

[0032] Skin biopsies from 6 patients with SSc and 8 patients with SLE were available for immunohistochemistry. They were all taken for diagnostic purposes in stages of active skin disease. Control biopsies were taken from unaffected skin of patients undergoing surgery.

[0033] Informed consent for participating in this study was obtained from all donors.

[0034] Recombinant Proteins

[0035] The entire coding sequence of the b isoform of human IFI16 (SEQ ID. No.:1, HIStagged IFI16; MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSSLMSV KMGKKYKNIVLLKGLEVINDYHFRMVKSLLSNDLKLNLKMREEYD KIQIADLMEE KFRGDAGLGKLIKIFEDIPTLEDLAETLKKEKLKVKGPALSRKRKKEVDATSPAP STSSTVKTEGAEATPGAQKRKKSTKEKAGPKGSKVSEEQTQPPSPAGAGMSTAMG RSPSPKTSLSAPPNTSSTENPKTVAKCQVTPRRNVLQKRPVIVKVLSTTKPFEYE TPEMEKKIMFHATVATQTQFFHVKVLNTSLKEKFNGKKIIIISDYLEYDSLLEVN EESTVSEAGPNQTFEVPNKIINRAKETLKIDILHKQASGNIVYGVFMLHKKTVNQ KTTIYEIQDDRGKMDVVGTGQCHNIPCEEGDKLQLFCFRLRKKNQMSKLISEMHS FIQIKKKTNPRNNDPKSMKLPQEQRQLPYPSEASTTFPESHLRTPQMPPTTPSSS FFTKKSEDTISKMNDFMRMQILKEGSHFPGPFMTSIGPAESHPHTPQMPPSTPSS SFLTTLKPRLKTEPEEVSIEDSAQSDLKEVMVLNATESFVYEPKEQKKMFHATVA TENEVFRVKVFNIDLKEKFTPKKIIAIANYVCRNGFLEVYPFTLVADVNADRNME IPKGLIRSASVTPKINQLCSQTKGSFVNGVFEVHKKNVRGEFTYYEIQDNTGKME VVVHGRLTTINCEEGDKLKLTCFELAPKSGNTGELRSVIHSHIKVIKTRKNKKDI LNPDSSMETSPDFFF) was subcloned from pBluescript in the pET30a expression vector (Novagen, Madison, USA), containing an N-terminal histidine tag. The sequences encoding N-terminal (SEQ ID No.:2, HIStagged-IFI16 N-term, amino acid residues 1-200, MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSLMSVK MGKKYKNIVLLKGLEVINDYHFRMVKSLLSNDLKLNLKMREEYDKIQIADLMEEK FRGDAGLGKLIKIFEDIPTLEDLAETLKKEKLKVKGPALSRKRKKEVDATSPAPS TSSTVKTEGAEATPGAQKRKKSTKEKAGPKGSKVSEEQTQPPSPAGAGMSTAMGR SPSPKTSLSAPPNTSSTENPKTVAKCQVTPRRNVL) or C-terminal (IFI16 C-term, residues 525-729, (SEQ ID No.:3 MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSMVLNA TESFVYEPKEQKKMFHATVATENEVFRVKVFNIDLKEKFTPKKI IAIANYVCRNG FLEVYPFTLVADVNADRNMEIPKGLIRSASVTPKINQLCSQTKGSFVNGVFEVHK KNVRGEFTYYEIQDNTGKMEVVVHGRLTTINCEEGDKLKLTCFELAPKSGNTGEL RSVIHSHIKVIKTRKNKKDILNPDSSMETSPDFFF) fragments of IFI16 were amplified by PCR and cloned in frame in the pET30a vector. Expression and affinity purification were performed following standard procedures. The purity of the proteins was assessed by Coomassie blue staining following 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). As negative controls for ELISA and immunoblotting, the polypeptide encoded by the pET30a empty vector (SEQ ID No.:4; control peptide, MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDK AMAD IGSEFELRRQACGRTRAPPPPPLRSGC) was expressed and purified with the same protocol.

[0036] Immunoblotting and Immunohistology

[0037] 250 ng of recombinant IFI16 (SEQ ID No.:1), IFI16-N-term (SEQ ID No.:2), IFI16 C-term (SEQ ID No.:3) or control peptide (SEQ ID No.:4) were separated using SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked in 3% Top Block (Fluka, St. Louis, USA) and then incubated with 1:100 dilution of the patients sera. After washing, the membranes were incubated with horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG (DakoCytomation, Glostrup, Denmark). The immunocomplexes were detected by enhanced chemiluminescence (ECL, GE Healthcare) and signals acquired with Versadoc3000 (Bio-Rad Laboratories Inc., Hercules, USA). After stripping, the membranes were reprobed with anti-IFI16 C-terminal rabbit polyclonal antibody (13) or anti-IFI16 N-terminal mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) to confirm the presence of the recombinant proteins.

[0038] Immunohistochemical analysis for IFI16 expression was performed on sections from paraffin-embedded tissues placed on silane-coated glass slides as previously described (6).

[0039] Determination of Antibody Titers Towards Human Recombinant IFI16 by ELISA

[0040] Polystyrene microwell plates for enzyme-linked immunoabsorbent assay (ELISA) (Nunc-Immuno MaxiSorp, Nunc, Roskilde, Denmark) were coated for 16 h at 4° C. with 2 μg/ml of either human recombinant IFI16 (SEQ ID No.:1) or control peptide solubilized in 0.2 ml of phosphate-buffered saline (PBS). After blocking with coating buffer containing 3% bovine serum albumin (BSA) in PBS, patients sera, diluted 1:100, were added in duplicate and incubated for 1 h at 37° C. After washing, HRP-conjugated rabbit anti-human IgG (dilution 1:4800) (DakoCytomation) was added and incubated for 1 h at 37° C. After substrate addition, absorbance was measured at 490 nm using a Bio-Rad microplate reader (Bio-Rad). The results were corrected by subtracting the background reactivity of the reference mixture.

[0041] Data Analysis and Statistical Calculations

[0042] Statistical analysis was performed by SPSS statistical software (SPSS Inc., Chicago, USA) using the one-way ANOVA with Bonferroni Multiple Comparisons tests. Either Fisher's exact test or chi-square test was used for measuring association. The independent effect of significant variables was assessed using forward conditional logistic regression. Positivity cut-off values were calculated as the 95^{th} percentiles in the control population. With substantial deviation

from normality, data was natural log transformed before parametric analysis was performed.

Results

Example 1

Anti-IFI16 Autoantibody Levels by ELISA and Skin IFI16 Immunoreactivity were Elevated in SSc and SLE

[0043] As recently reported in the normal epidermis from healthy controls, IFI16 expression was restricted to the basal layer (FIG. **1**A). Notably, as shown in the representative sections in FIG. **1**, IFI16 expression was greatly increased and found ubiquitously expressed in all layers of the epidermis in the lesional skin from both SSc (FIG. **1**B) and SLE (FIG. **1**C) patients. Furthermore, the dermal inflammatory infiltrate showed IFI16 positive staining, indicating that it is expressed to a high level in lymphocytes, fibroblasts and endothelial cells.

[0044] To verify whether the increased expression of IFI16 in the affected skin was related to the presence of anti-IFI16 autoantibodies, their presence and levels in serum samples from 100 SLE patients and 82 SSc patients were assessed using ELISA with recombinant IFI16 protein. Other autoimmune diseases, including SjS, RA and CU as well as healthy individuals as normal controls were also examined. In addition, to further assess the clinical specificity of anti-IFI16 autoantibodies, 40 sera from patients with chronic HCV infection were also subjected to anti-IFI16 ELISA. Absorbance values higher than the 95^{th} percentile of the control population (0.360) were considered to be positive in this study. As depicted in FIG. **2**A, patients with SSc, SLE and SjS exhibited significantly (one-way ANOVA $p<0.0001$) higher anti-IFI16 antibody levels of the IgG isotype than normal controls did (SSc $p<0.0005$, SLE $p<0.002$ and SjS $p<0.001$ respectively). Anti-IFI16 titers above the 95^{th} percentile of the controls were observed in 21% of SSc, 26% of SLE and 50% of SjS respectively. By contrast, anti-IFI16 prevalence was 4% in RA, 5% in CU and 10% in HCV patients respectively. Thus, IgG autoantibody levels were increased in SLE, SjS and SSc but not in other autoimmune diseases, including RA and CU or in patients with increased polyclonal reactivity such as HCV-positive patients.

Example 2

Association of anti-IFI16 Antibodies with Clinical Parameters

[0045] Since the presence of anti-IFI16 autoantibodies had already been reported in both SjS and SLE, where a significant serological heterogeneity is well known to occur (14), but not in SSc, a decision was taken to gain more insight into the actual role of anti-IFI16 autoantibodies in SSc pathogenesis. Univariate analysis showed that anti-IFI16 autoreactivity in SSc patients was not associated with either disease duration or disease severity as measured by Medsger stage, HAQ disability index, organ involvement and positivity to other autoantibodies (data not shown). By contrast, a strict association between anti-IFI16 reactivity and the cutaneous form of the disease was found, with patients in the limited cutaneous scleroderma (lc-SSc) having higher anti-IFI16 IgG titers than patients with the diffuse form (dc-SSc) ($p=0.017$). Indeed, as shown in FIG. **2**B, anti-IFI16 titers above the 95^{th} percentile of the controls were observed in 28% of patients with lc-SSc but only in 4% of dc-SSc (95% confidence interval for the difference 5-37%).

[0046] In line with previous reports, the presence or titers of anti-IFI16 antibodies did not correlate with clinical manifestations or disease activity in both SLE and SjS patients (data not shown).

Example 3

Logistic Regression Model and Sensitivity-Specificity Analysis for lc-SSc Prediction

[0047] In our samples the cutaneous form of SSc was significantly associated with both anti-centromere ($\chi^2=18.771$ $p<0.0005$) and anti-topoisomerase ($\chi^2=32.689$ $p<0.0005$) autoantibodies. Logistic regression showed that all the three serological markers were independent predictors of the cutaneous form of scleroderma, and their combination was able to explain 62% of the associated variability. This model was able to correctly predict 89% of the clinical presentation forms of scleroderma. Moreover, anti-IFI16 reactivity displayed lower sensitivity (28%) and higher specificity (96%) than those found with either anti-topoisomerase I (95% and 67% respectively) or anti-centromere (65% and 92% respectively). The combined use of anti-IFI16 and anti-centromere markers gave rise to the highest sensitivity and specificity score (79% and 92% respectively). Interestingly, in the SSc subgroup negative for both anti-centromere and anti-topoisomerase I reactivity, all patients with anti-IFI16 positivity displayed the limited cutaneous form of SSc (specificity 100% and positive predictive value 100%).

Example 4

Immunoblotting Analysis for anti-IFI16 Antibody

[0048] The presence of anti-IFI16 antibodies was also evaluated by immunoblotting analysis using recombinant IFI16 protein either full length or deleted fragments as schematically represented in FIG. **3**A. Western blots were performed on 25 SSc sera, 17 from patients with anti-IFI16 titers above and 8 with titers below the 95^{th} percentile of the controls. As illustrated in the representative immunoblotting in FIG. **35**, low-titer sera did not exhibit reactivity with IFI16, thus confirming the specificity of the ELISA technique. By contrast, only 10 of the 17 IFI16 high-titer sera were positive, very likely because immunoblot detects antibodies directed against linear epitopes while ELISA either linear and conformational epitopes. Correlation between anti-IFI16 autoantibody titers and the intensity of immunoreactive bands was not depicted.

[0049] To further characterize the antigenic specificity of the IFI16 positive sera, the 10 sera recognizing linear epitopes were analyzed for their reactivity against the N-terminal (IFI16 N-term) and C-terminal (IFI16 C-term) fragments of IFI16 respectively. As shown in the representative immunoblot in FIG. **3**C, 4 sera displayed reactivity against the N-terminal fragment, 3 against the C-terminal fragment, 2 recognized both fragments and 1 displayed no reaction.

[0050] All together these data suggest a polyclonal immune response against IFI16 in patients with SSc.

Example 5

Anti-IFI16 Autoantibodies in Patients Affected by Multiple Sclerosis

[0051] The presence of anti-IFI16 autoantibodies and their levels in serum samples from 163 patients affected by mul-

tiple sclerosis (MS) and 64 healthy individuals as normal controls were examined by ELISA with recombinant IFI16 protein. As shown in FIG. **4**, patients with MS exhibited significantly (unpaired t-test $p<0.05$) higher anti-IFI16 antibody levels of the IgG isotype than normal controls did.

[0052] Naturally, while the principle of the invention remains the same, the details of construction and the embodiments may widely vary with respect to what has been described and illustrated purely by way of example, without departing from the scope of the present invention as defined in the appended claims.

REFERENCES

[0053] 1. Landolfo S, Gariglio M, Gribaudo G, Lembo D. The Ifi 200 genes: an emerging family of IFN-inducible genes. Biochimie 1998; 80 (8-9):721-8.

[0054] 2. Ludlow L E, Johnstone R W, Clarke C J. The HIN-200 family: more than interferon-inducible genes? Exp Cell Res 2005; 308(1):1-17.

[0055] 3. Rozzo S J, Allard J D, Choubey D, Vyse T J, Izui S, Peltz G, et al. Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. Immunity 2001; 15(3):435-43.

[0056] 4. Seelig H P, Ehrfeld H, Renz M. Interferon-gamma-inducible protein p16. A new target of antinuclear antibodies in patients with systemic lupus erythematosus. Arthritis Rheum 1994; 37(11):1672-83.

[0057] 5. Uchida K, Akita Y, Matsuo K, Fujiwara S, Nakagawa A, Kazaoka Y, et al. Identification of specific autoantigens in Sjogren's syndrome by SEREX. Immunology 2005; 116(1):53-63.

[0058] 6. Gariglio M, Azzimonti B, Pagano M, Palestro G, De Andrea M, Valente G, et al. Immunohistochemical expression analysis of the human interferon-inducible gene IFI16, a member of the HIN200 family, not restricted to hematopoietic cells. J Interferon Cytokine Res 2002; 22(7):815-21.

[0059] 7. Calamia K T, Balabanova M. Vasculitis in systemic lupus erythematosis. Clin Dermatol 2004; 22(2): 148-56.

[0060] 8. Raffaella R, Gioia D, De Andrea M, Cappello P, Giovarelli M, Marconi P, et al. The interferon-inducible IFI16 gene inhibits tube morphogenesis and proliferation of primary, but not HPV16 E6/E7-immortalized human endothelial cells. Exp Cell Res 2004; 293(2):331-45.

[0061] 9. LeRoy E C, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger T A, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988; 15(2):202-5.

[0062] 10. Valentini G, Medsger T A, Jr., Silman A J, Bombardieri S. Conclusion and identification of the core set of variables to be used in clinical investigations. Clin Exp Rheumatol 2003; 21(3 Suppl 29):S47-8.

[0063] 11. Medsger T A, Jr., Silman A J, Steen V D, Black C M, Akesson A, Bacon P A, et al. A disease severity scale for systemic sclerosis: development and testing. J Rheumatol 1999; 26(10):2159-67.

[0064] 12. Steen V D, Medsger T A, Jr. The value of the Health Assessment Questionnaire and special patient-generated scales to demonstrate change in systemic sclerosis patients over time. Arthritis Rheum 1997; 40(11):1984-91.

[0065] 13. Gugliesi F, Mondini M, Ravera R, Robotti A, de Andrea M, Gribaudo G, et al. Up-regulation of the interferon-inducible IFI16 gene by oxidative stress triggers p53 transcriptional activity in endothelial cells. J Leukoc Biol 2005; 77(5):820-9.

[0066] 14. Sherer Y, Gorstein A, Fritzler M J, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. Semin Arthritis Rheum 2004; 34(2):501-37.

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Ser Pro Asp Leu Gly Thr Asp Asp Asp Asp Lys Ala Met Ala Asp Ile
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Gly Ser Ser Leu Met Ser Val Lys Met Gly Lys Lys Tyr Lys Asn Ile
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Val Leu Leu Lys Gly Leu Glu Val Ile Asn Asp Tyr His Phe Arg Met
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Arg Gly Asp Ala Gly Leu Gly Lys Leu Ile Lys Ile Phe Glu Asp Ile
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Pro Thr Leu Glu Asp Leu Ala Glu Thr Leu Lys Lys Glu Lys Leu Lys
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Val Lys Gly Pro Ala Leu Ser Arg Lys Arg Lys Lys Glu Val Asp Ala
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Thr Ser Pro Ala Pro Ser Thr Ser Ser Thr Val Lys Thr Glu Gly Ala
                165                 170                 175

Glu Ala Thr Pro Gly Ala Gln Lys Arg Lys Lys Ser Thr Lys Glu Lys
            180                 185                 190

Ala Gly Pro Lys Gly Ser Lys Val Ser Glu Glu Gln Thr Gln Pro Pro
        195                 200                 205

Ser Pro Ala Gly Ala Gly Met Ser Thr Ala Met Gly Arg Ser Pro Ser
    210                 215                 220

Pro Lys Thr Ser Leu Ser Ala Pro Pro Asn Thr Ser Ser Thr Glu Asn
225                 230                 235                 240

Pro Lys Thr Val Ala Lys Cys Gln Val Thr Pro Arg Arg Asn Val Leu
                245                 250                 255

Gln Lys Arg Pro Val Ile Val Lys Val Leu Ser Thr Thr Lys Pro Phe
            260                 265                 270

Glu Tyr Glu Thr Pro Glu Met Glu Lys Lys Ile Met Phe His Ala Thr
        275                 280                 285

Val Ala Thr Gln Thr Gln Phe Phe His Val Lys Val Leu Asn Thr Ser
    290                 295                 300

Leu Lys Glu Lys Phe Asn Gly Lys Lys Ile Ile Ile Ile Ser Asp Tyr
305                 310                 315                 320

Leu Glu Tyr Asp Ser Leu Leu Glu Val Asn Glu Glu Ser Thr Val Ser
                325                 330                 335

Glu Ala Gly Pro Asn Gln Thr Phe Glu Val Pro Asn Lys Ile Ile Asn
            340                 345                 350

Arg Ala Lys Glu Thr Leu Lys Ile Asp Ile Leu His Lys Gln Ala Ser
        355                 360                 365

Gly Asn Ile Val Tyr Gly Val Phe Met Leu His Lys Lys Thr Val Asn
    370                 375                 380

Gln Lys Thr Thr Ile Tyr Glu Ile Gln Asp Asp Arg Gly Lys Met Asp
385                 390                 395                 400

Val Val Gly Thr Gly Gln Cys His Asn Ile Pro Cys Glu Glu Gly Asp
                405                 410                 415

Lys Leu Gln Leu Phe Cys Phe Arg Leu Arg Lys Lys Asn Gln Met Ser
            420                 425                 430

Lys Leu Ile Ser Glu Met His Ser Phe Ile Gln Ile Lys Lys Lys Thr
        435                 440                 445

Asn Pro Arg Asn Asn Asp Pro Lys Ser Met Lys Leu Pro Gln Glu Gln
    450                 455                 460

Arg Gln Leu Pro Tyr Pro Ser Glu Ala Ser Thr Thr Phe Pro Glu Ser
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His Leu Arg Thr Pro Gln Met Pro Pro Thr Thr Pro Ser Ser Ser Phe
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Arg Met Gln Ile Leu Lys Glu Gly Ser His Phe Pro Gly Pro Phe Met
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Thr Ser Ile Gly Pro Ala Glu Ser His Pro His Thr Pro Gln Met Pro
    530                 535                 540

Pro Ser Thr Pro Ser Ser Ser Phe Leu Thr Thr Leu Lys Pro Arg Leu
545                 550                 555                 560

Lys Thr Glu Pro Glu Glu Val Ser Ile Glu Asp Ser Ala Gln Ser Asp
                565                 570                 575

Leu Lys Glu Val Met Val Leu Asn Ala Thr Glu Ser Phe Val Tyr Glu
            580                 585                 590

Pro Lys Glu Gln Lys Lys Met Phe His Ala Thr Val Ala Thr Glu Asn
        595                 600                 605

Glu Val Phe Arg Val Lys Val Phe Asn Ile Asp Leu Lys Glu Lys Phe
    610                 615                 620

Thr Pro Lys Lys Ile Ile Ala Ile Ala Asn Tyr Val Cys Arg Asn Gly
625                 630                 635                 640

Phe Leu Glu Val Tyr Pro Phe Thr Leu Val Ala Asp Val Asn Ala Asp
                645                 650                 655

Arg Asn Met Glu Ile Pro Lys Gly Leu Ile Arg Ser Ala Ser Val Thr
            660                 665                 670

Pro Lys Ile Asn Gln Leu Cys Ser Gln Thr Lys Gly Ser Phe Val Asn
        675                 680                 685

Gly Val Phe Glu Val His Lys Lys Asn Val Arg Gly Glu Phe Thr Tyr
    690                 695                 700

Tyr Glu Ile Gln Asp Asn Thr Gly Lys Met Glu Val Val Val His Gly
705                 710                 715                 720

Arg Leu Thr Thr Ile Asn Cys Glu Glu Gly Asp Lys Leu Lys Leu Thr
                725                 730                 735

Cys Phe Glu Leu Ala Pro Lys Ser Gly Asn Thr Gly Glu Leu Arg Ser
            740                 745                 750

Val Ile His Ser His Ile Lys Val Ile Lys Thr Arg Lys Asn Lys Lys
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Phe
785


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Ser Pro Asp Leu Gly Thr Asp Asp Asp Asp Lys Ala Met Ala Asp Ile
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Leu Leu Lys Gly Leu Glu Val Ile Asn Asp Tyr His Phe Arg Met Val
65                  70                  75                  80

Lys Ser Leu Leu Ser Asn Asp Leu Lys Leu Asn Leu Lys Met Arg Glu
                85                  90                  95

Glu Tyr Asp Lys Ile Gln Ile Ala Asp Leu Met Glu Glu Lys Phe Arg
            100                 105                 110

Gly Asp Ala Gly Leu Gly Lys Leu Ile Lys Ile Phe Glu Asp Ile Pro
        115                 120                 125

Thr Leu Glu Asp Leu Ala Glu Thr Leu Lys Lys Glu Lys Leu Lys Val
    130                 135                 140

Lys Gly Pro Ala Leu Ser Arg Lys Arg Lys Lys Glu Val Asp Ala Thr
145                 150                 155                 160

Ser Pro Ala Pro Ser Thr Ser Ser Thr Val Lys Thr Glu Gly Ala Glu
                165                 170                 175

Ala Thr Pro Gly Ala Gln Lys Arg Lys Lys Ser Thr Lys Glu Lys Ala
            180                 185                 190

Gly Pro Lys Gly Ser Lys Val Ser Glu Glu Gln Thr Gln Pro Pro Ser
        195                 200                 205

Pro Ala Gly Ala Gly Met Ser Thr Ala Met Gly Arg Ser Pro Ser Pro
    210                 215                 220

Lys Thr Ser Leu Ser Ala Pro Pro Asn Thr Ser Ser Thr Glu Asn Pro
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            20                  25                  30

Ser Pro Asp Leu Gly Thr Asp Asp Asp Asp Lys Ala Met Ala Asp Ile
        35                  40                  45

Gly Ser Met Val Leu Asn Ala Thr Glu Ser Phe Val Tyr Glu Pro Lys
    50                  55                  60

Glu Gln Lys Lys Met Phe His Ala Thr Val Ala Thr Glu Asn Glu Val
65                  70                  75                  80

Phe Arg Val Lys Val Phe Asn Ile Asp Leu Lys Glu Lys Phe Thr Pro
                85                  90                  95

Lys Lys Ile Ile Ala Ile Ala Asn Tyr Val Cys Arg Asn Gly Phe Leu
            100                 105                 110

Glu Val Tyr Pro Phe Thr Leu Val Ala Asp Val Asn Ala Asp Arg Asn
        115                 120                 125

Met Glu Ile Pro Lys Gly Leu Ile Arg Ser Ala Ser Val Thr Pro Lys
    130                 135                 140
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Ile Asn Gln Leu Cys Ser Gln Thr Lys Gly Ser Phe Val Asn Gly Val
145                 150                 155                 160

Phe Glu Val His Lys Lys Asn Val Arg Gly Glu Phe Thr Tyr Tyr Glu
                165                 170                 175

Ile Gln Asp Asn Thr Gly Lys Met Glu Val Val Val His Gly Arg Leu
            180                 185                 190

Thr Thr Ile Asn Cys Glu Glu Gly Asp Lys Leu Lys Leu Thr Cys Phe
        195                 200                 205

Glu Leu Ala Pro Lys Ser Gly Asn Thr Gly Glu Leu Arg Ser Val Ile
    210                 215                 220

His Ser His Ile Lys Val Ile Lys Thr Arg Lys Asn Lys Lys Asp Ile
225                 230                 235                 240

Leu Asn Pro Asp Ser Ser Met Glu Thr Ser Pro Asp Phe Phe Phe
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            20                  25                  30

Ser Pro Asp Leu Gly Thr Asp Asp Asp Asp Lys Ala Met Ala Asp Ile
        35                  40                  45

Gly Ser Glu Phe Glu Leu Arg Arg Gln Ala Cys Gly Arg Thr Arg Ala
    50                  55                  60

Pro Pro Pro Pro Pro Leu Arg Ser Gly Cys
65                  70
```

1. (canceled)

2. Method according to claim **4**, wherein the reference mixture is represented by a control peptide, particularly peptide SEQ ID NO:4.

3. Method according to claim **4**, characterized in that said autoimmunoassay is carried out on whole blood, serum, plasma, saliva, tears, sweat, or synovial fluid.

4. Method of differential diagnosis of limited cutaneous form of scleroderma (lc-SSc) in a subject suspected of or at risk of having an autoimmune disease, wherein autoantibodies against IFI16 protein, fragments or peptides thereof are detected by an ELISA and wherein the results are corrected by subtracting the background reactivity of a reference mixture.

5. Method according to claim **4**, characterized in that a difference between the level of said autoantibodies against IFI16 protein fragments or peptides thereof in the subject and a normal level of said autoantibodies is detected.

6. Method according to claim **4**, characterized in that the diagnosis is based on the detection of a higher level of autoantibodies against IFI16 protein, fragments or peptides thereof in the subject as compared to the normal level of said autoantibodies.

7. Method according to claim **4**, characterized in that the diagnosis is based on the detection of a higher level of autoantibodies against IFI16 protein, fragments or peptides thereof in the subject as compared to a level of said autoantibodies in patients suffering of a diffuse cutaneous form of scleroderma (dc-SSc).

8. Method according to claim **4**, characterized in that it comprises the following steps:

a) contacting a sample from a patient with an antigen comprising IFI16 protein, fragments or peptides thereof to form a complex of the autoantibody and the antigen;

b) detecting the presence of the autoantibody antigen complex.

9. Method according to claim **8**, characterized in that the sample is selected from the group consisting of whole blood, serum, plasma, saliva, tears, sweat, or synovial fluid.

10. Method according to claim **8**, characterized in that the antigen is of human, animal, synthetic or recombinant origin.

11. Method according to claim **8**, characterized in that the antigen is bound to a solid carrier.

12. Method according to claim **8**, characterized in that the detection is performed using an anti-human IgG antibody.

13. Method according to claim **12**, characterized in that the anti-human IgG antibody is conjugated to a detectable marker.

14. Method according to claim **13**, characterized in that the detectable marker is selected from horseradish peroxidase, alkaline phosphatase, biotin or fluorescent dyes.

15. Kit for the determination of anti-IFI16 autoantibodies in a sample including a plate of wells having bound thereto IFI16 protein, fragments or peptides thereof, and wells having bound thereto a reference mixture and a detection reagent.

16. Kit according to claim **15**, characterized in that the detection reagent is an anti-human IgG antibody conjugated to a detectable marker.

17. Kit according to claim **16**, characterized in that the detectable marker is selected from horseradish peroxidase, alkaline phosphatase, biotin or fluorescent dyes.

18. Method of differential diagnosis of limited cutaneous form of scleroderma (lc-SSc) in a subject suspected of or at risk of having an autoimmune disease, wherein a combination of autoantibodies against IFI16 protein, fragments or peptides thereof with anti-centromere autoantibodies is detected.

19. Method of differential diagnosis of limited cutaneous form of scleroderma (lc-SSc) in a subject suspected of or at risk of having an autoimmune disease, wherein a combination of autoantibodies against IFI16 protein, fragments or peptides thereof with anti-centromere autoantibodies and anti-topoisomerase I autoantibodies are detected.

20. Method of diagnosis of multiple sclerosis in a subject, wherein autoantibodies against IFI16 protein, fragments or peptides thereof are detected.

* * * * *

专利名称(译)	硬皮病的鉴别诊断		
公开(公告)号	US20100105086A1	公开(公告)日	2010-04-29
申请号	US12/438199	申请日	2007-08-20
[标]申请(专利权)人(译)	LANDOLFO SANTO MONDINI MICHELE GARIGLIO MARISA		
申请(专利权)人(译)	LANDOLFO SANTO MONDINI MICHELE GARIGLIO MARISA		
当前申请(专利权)人(译)	LANDOLFO SANTO MONDINI MICHELE GARIGLIO MARISA		
[标]发明人	LANDOLFO SANTO MONDINI MICHELE GARIGLIO MARISA		
发明人	LANDOLFO, SANTO MONDINI, MICHELE GARIGLIO, MARISA		
IPC分类号	G01N33/535		
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优先权	102006901441995 2006-08-21 IT		
外部链接	Espacenet USPTO		

摘要(译)

使用IFI16蛋白，其片段或肽用于在怀疑患有自身免疫疾病或有患自身免疫疾病风险的受试者中鉴别诊断有限的皮肤形式的硬皮病（lc-SSc）和相应的诊断方法和试剂盒。

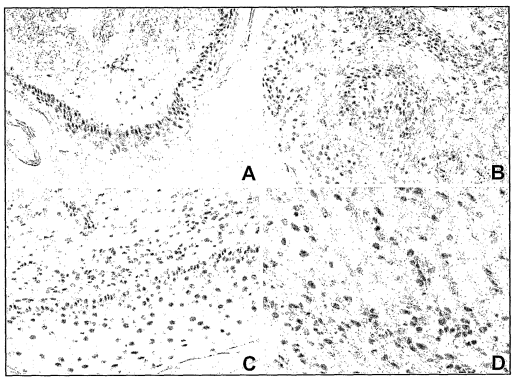

FIG. 1