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Authors

Upadhyay, Vaibhav
Yoon, Young
Vazquez, Sara
et al.

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Phage Immunoprecipitation-Sequencing Reveals CDHR5 Autoantibodies in Select Patients With Interstitial Lung Disease

Vaibhav Upadhyay,[1] Young me Yoon,[2] Sara E. Vazquez,[3] Tania E. Velez,[4] Kirk D. Jones,[1] Cathryn T. Lee,[2] Christopher S. Law,[1] Paul J. Wolters,[1] Seoyeon Lee,[1] Monica M. Yang,[1] Erica Farrand,[1] Imre Noth,[5] Mary E. Strek,[2] Mark S. Anderson,[1] Joseph L. DeRisi,[3] Anne I. Sperling,[4] and Anthony K. Shum[1]

Objective. Interstitial lung diseases (ILDs) are a heterogeneous group of disorders that can develop in patients with connective tissue diseases. Establishing autoimmunity in ILD impacts prognosis and treatment. Patients with ILD are screened for autoimmunity by measuring antinuclear autoantibodies, rheumatoid factors, and other nonspecific tests. However, this approach may miss autoimmunity that manifests as autoantibodies to tissue antigens not previously defined in ILD.

Methods. We use Phage Immunoprecipitation-Sequencing (PhIP-Seq) to conduct an autoantibody discovery screen of patients with ILD and controls. We screened for novel autoantigen candidates using PhIP-Seq. We next developed a radio-labeled binding assay and validated the leading candidate in 398 patients with ILD recruited from two academic medical centers and 138 blood bank individuals that formed our reference cohort.

Results. PhIP-Seq identified 17 novel autoreactive targets, and machine learning classifiers derived from these targets discriminated ILD serum from controls. Among the 17 candidates, we validated CDHR5 and found CDHR5 autoantibodies in patients with rheumatologic disorders and importantly, patients not previously diagnosed with autoimmunity. Using survival and transplant free–survival data available from one of the two centers, patients with CDHR5 autoantibodies showed worse survival compared with other patients with connective tissue disease ILD.

Conclusion. We used PhIP-Seq to define a novel CDHR5 autoantibody in a subset of select patients with ILD. Our data complement a recent study showing polymorphisms in the CDHR5-IRF7 gene locus strongly associated with titer of anticentromere antibodies in systemic sclerosis, creating a growing body of evidence suggesting a link between CDHR5 and autoimmunity.

INTRODUCTION

Interstitial lung diseases (ILDs) are a heterogeneous group of disorders with well-defined disease associations and genetic underpinnings.[1–4] Connective tissue disorders (also referred to as systemic rheumatic disorders), such as rheumatoid arthritis (RA) and scleroderma, are known causes of ILD.[5] In patients with a connective tissue disease (CTD), ILD often has the greatest impact on morbidity and mortality,[3,6] Detecting whether autoimmunity is present in a patient with ILD has important implications for treatment and prognosis, although identifying autoimmunity can be difficult in patients that do not meet the criteria of a known CTD.[5]

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[1]Vaibhav Upadhyay, MD, PhD, Kirk D. Jones, MD, Christopher S. Law, BS, Paul J. Wolters, MD, Seoyeon Lee, BA, Monica M. Yang, MD, Erica Farrand, MD, Mark S. Anderson, MD, PhD, Anthony K. Shum, MD: University of California San Francisco; [2]Young me Yoon, PhD, Cathryn T. Lee, MD, Mary E. Strek, MD: University of Chicago, Illinois; [3]Sara E. Vazquez, PhD, Joseph L. DeRisi, PhD: University of California San Francisco and Chan Zuckerberg Biohub; [4]Tania E. Velez, PhD, Anne I. Sperling, PhD: University of Chicago, Illinois, and University of Virginia, Charlottesville; [5]Imre Noth, MD: University of Virginia, Charlottesville.

Drs Upadhyay and Yoon are co-first authors and contributed equally to this work.

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Address correspondence via email to Anne I. Sperling, PhD, at anne.sperling@virginia.edu; or to Anthony K. Shum, MD, at anthony.shum@ucsf.edu.

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Expert guidelines recommend that every patient with ILD should be screened for a CTD by measuring serologic markers.[4,5,7] However, there is no consensus as to which tests should be performed.[4] Many of the laboratory studies recommended (antinuclear antibodies, C-reactive protein, erythrocyte sedimentation rate, rheumatoid factor) lack specificity, and their use for the detection of autoimmunity in the context of ILD has not been rigorously studied.[4,5]

Rheumatologists and pulmonologists have recognized for years that autoimmune-associated ILD may in fact be a unique disease.[8,9] Importantly, ILD is not considered a criterion for nearly all the defined CTDs in which it manifests. There is a well-described subset of patients with ILD with clinical features of autoimmunity that do not meet criterion for a defined CTD.[7] Thus, it is possible that for some patients with ILD, there are autoantibodies unique to ILD that are missed by standard laboratory tests used to assess for an underlying rheumatologic condition.

Furthermore, detecting autoantibodies outside the context of a defined CTD is increasingly relevant.[10,11] Recent data show that such autoantibodies can impact patient outcomes, even in clinical settings in which autoimmune mechanisms are not the primary driver of disease. In COVID-19, autoantibodies to type 1 interferons predispose individuals to severe disease, including significant lung damage.[12–14] Thus, it is possible that novel tissue autoantibodies in patients with ILD, including in those without a defined CTD, may have an important role in disease.

Phage Immunoprecipitation-Sequencing (PhIP-Seq) is an emerging technology enabling massively parallel profiling of autoreactive antibodies in patient serum by pairing programmable phage-display libraries with next generation sequencing.[15–18] PhIP-Seq profiles genes and identifies autoantigen candidates, which can then be validated as autoantibodies using an orthogonal approach, such as radioligand binding assay (RLBA). PhIP-Seq facilitates the profiling of serologically reactive peptide epitopes tiling all open-reading frames of the human genome. PhIP-Seq has been used to define novel autoantibodies in established diseases, provided insight into pathogenic mechanisms, and established new autoimmune syndromes.[15,19] PhIP-Seq has also been recently applied to patients with clinical syndromes of unclear etiology including long COVID[20] and has identified microbial reactive epitopes in inflammatory bowel disease.[21] We hypothesized that ILDs, which are heterogeneous and require intense clinical assessment of autoimmunity, would present a valuable application of PhIP-Seq to discover novel autoantibodies in ILD not revealed by standard tests.

MATERIALS AND METHODS

Study design. Patient samples were obtained from two academic medical centers with established ILD programs, including regular presentation of patients at a multidisciplinary conference. Diagnoses for patients with ILD were classified based on multidisciplinary discussion, including detailed clinical, radiographic, and pathologic information. Biobanked serum was collected and stored by investigators at each site through established research protocols at each center (AKS, PW at the University of California San Francisco [UCSF] and AS, IN, MS at University of Chicago). All research patients had samples drawn as described under institutional review board (IRB) approval (UCSF IRB 10-02467, UCSF IRB 10-01592, & University of Chicago 14163A-AM059).

Two authors (VU and CTL) collapsed categorization of ILD to shared data categories to facilitate data merging while maintaining the integrity of the original diagnosis. The original center labels and shared diagnostic categories for this manuscript are described in Supplementary Table 1A. Control samples were collected from multiple laboratories and represented blood banked donors in San Francisco and New York. Reference samples were collected, aligned, and processed as described prev,[16,17] and serum from these reference samples were rerun with the entire ILD cohort included in this study. A subset of samples of patients with RA without known ILD were run, and additional samples of patients without known ILD were available from the University of Chicago cohort. Chronic inflammatory disease is established in patients with RA from this cohort; the remaining were a combination of relatives of patients with ILD not known to have ILD themselves (n = 16) in whom other diseases were not ruled out and organ donors (n = 15). All samples not in the reference group of 138 samples contributed to candidate selection if their Z scores were above the cutoff. Self-identified race and ethnicity, biologic sex, and age ranges for screened patients are described in Table 1. Specific metadata assignments, including shared diagnostic label, age, sex, and self-identified race and ethnicity, are in Supplementary Table 1B.

The study as described is part of a two-institution collaboration. The work in this manuscript features PhIP-Seq data, which are also being used as a minor part of a separate publication for which a preprint was available at the time of the review process.[22]

PhIP-Seq protocol and analysis. The PhIP-Seq phage-display system was conducted as previously described.[16,17] Human serum was incubated with 10^{10} plaque forming units of a phage-display library tiling all open-reading frames of the human genome, which was incubated overnight, and precipitated using protein A/G magnetic beads, and used to infect *E. coli* for selective amplification of infective phage and next generation sequencing.[16,17] Some samples had sufficient serum available and arrayed for multiple technical replicates; where multiple technical replicates were performed, the maximum PhIP-Seq value collapsed to gene annotations was selected for candidate autoantigen selection though technical replicates were used in the machine learning classifier. Candidate autoantigens were selected by identifying genes with PhIP-Seq Z scores greater than 17 in each cohort and excluding genes in the blood bank control

Table 1. Demographic information for study patients*

Diagnosis or group		Patients (n)	Samples (n)
Control		138	150
Center 2 non-ILD[a]		46	46
Connective tissue disease ILD		80	104
Hypersensitivity pneumonitis		70	87
Idiopathic pulmonary fibrosis		102	136
Interstitial lung abnormality[b]		10	12
Other[c]		81	97
Unclassifiable		28	34
Unknown connective tissue disease ILD		27	38
Total		582	704
Self-identified race and ethnicity	Sex	Total screened	Age (mean ± SD)
White	Female	137	63.3 ± 11.9
	Male	170	67.0 ± 11.6
Black	Female	42	61.4 ± 11.0
	Male	8	53.6 ± 15.1
Latino/a/x[d]	Female	22	58.5 ± 13.3
	Male	14	66.2 ± 11.1
Unknown[e]	Female	3	70 ± 11.4
	Male	5	68.2 ± 9.8
	NA	9	82
Asian	Female	16	59.4 ± 12.0
	Male	13	68.5 ± 9.8
American Indian	Female	1	77 ± NA
	Male	2	68 ± 0
American Indian/Latino/a/x[f]	Male	1	63 ± NA
Other identity[g]	Male	1	63 ± NA

*ILD, interstitial lung disease; NA, not available.
[a]A subset of individuals without ILD were screened who had rheumatoid arthritis (n = 15) were the relative of patients with ILD who did not have ILD but in whom other diseases were not ruled out (n = 16 patients), and organ donors (n = 15 patients) were included from Center 2.
[b]Interstitial lung abnormality reflected a diagnosis in which underlying lung abnormalities were present (eg, broncheictasis or inflammation) though not felt to reflect ILD.
[c]Other reflected a group of diagnoses not otherwise represented on this table and were a heterogeneous group of disorders (eg, sarcoidosis, asbestosis etc).
[d]Latino/a/x indicated individuals at Center 1 that did not self-identify with race but identified with ethnicity as Hispanic or Latino at the Center 1 site or Hispanic at the Center 2 site.
[e]Indivduals without known race or ethnicity information were categorized as Unknown. One individual that did not specify race and ethnicity or sex was 82 years old.
[f]One individual identified as American Indian and Latino/a/x.
[g]Data on race, ethnicity, age, and sex were not available for 138 patients used as the screening reference group.

samples (n = 138 patients) that had any Z scores greater than 17. Candidates were further filtered as being found 10 times between both cohorts and found at least once in each cohort. Previously described autoantigens were identified via a literature review, and PhIP-Seq data for those candidates were plotted. Resulting Z score data for genes determined to be autoantigens was used to construct two random forest classifiers with R version 4.1.1, tidyverse (tidyverse 1.3.1), tidymodels (tidymodels 0.1.4), vip (vip 0.3.2), and pROC (pROC 1.18.0). UCSF is "Center 1" and University of Chicago is "Center 2" for the purposes of this manuscript. For the intercenter classifier, the blood bank control samples were separated into two groups and paired with one or the other cohort, and confidence intervals are displayed from pROC. "Not ILD" was a classification only used at Center 1 and is visually represented in all main text figures but excluded from the machine learning classifier training given it was not a used category at both centers (Figure 1). The "Not ILD" categorization was recoded to interstitial lung abnormality (ILA) for this manuscript after chart review by AKS. The classifier was generated on 169 control patients and included 12 technical replicates to reduce numeric differences between the ILD and control groups for a total of 181 control samples. Network analysis was conducted using identified candidate antigens as the Z score cutoff for screening candidate antigens and using a Z score cutoff of 50 for peptide data. A function was created in R to define co-occurrence associations by patient designation for Z score transformed PhIP-Seq data, and the resulting plot was created with the Igraph package (igraph 1.3.1).

Predicted protein structure. Alignments of 11 CDHR5 peptides from Supplementary Table 2 was completed using DECIPHER. Alpha fold (https://alphafold.ebi.ac.uk/) was employed to visualize predicted secondary structure for this protein using the CDHR5 sequence from Uniprot (accession A0A7L0FKT8).[23]

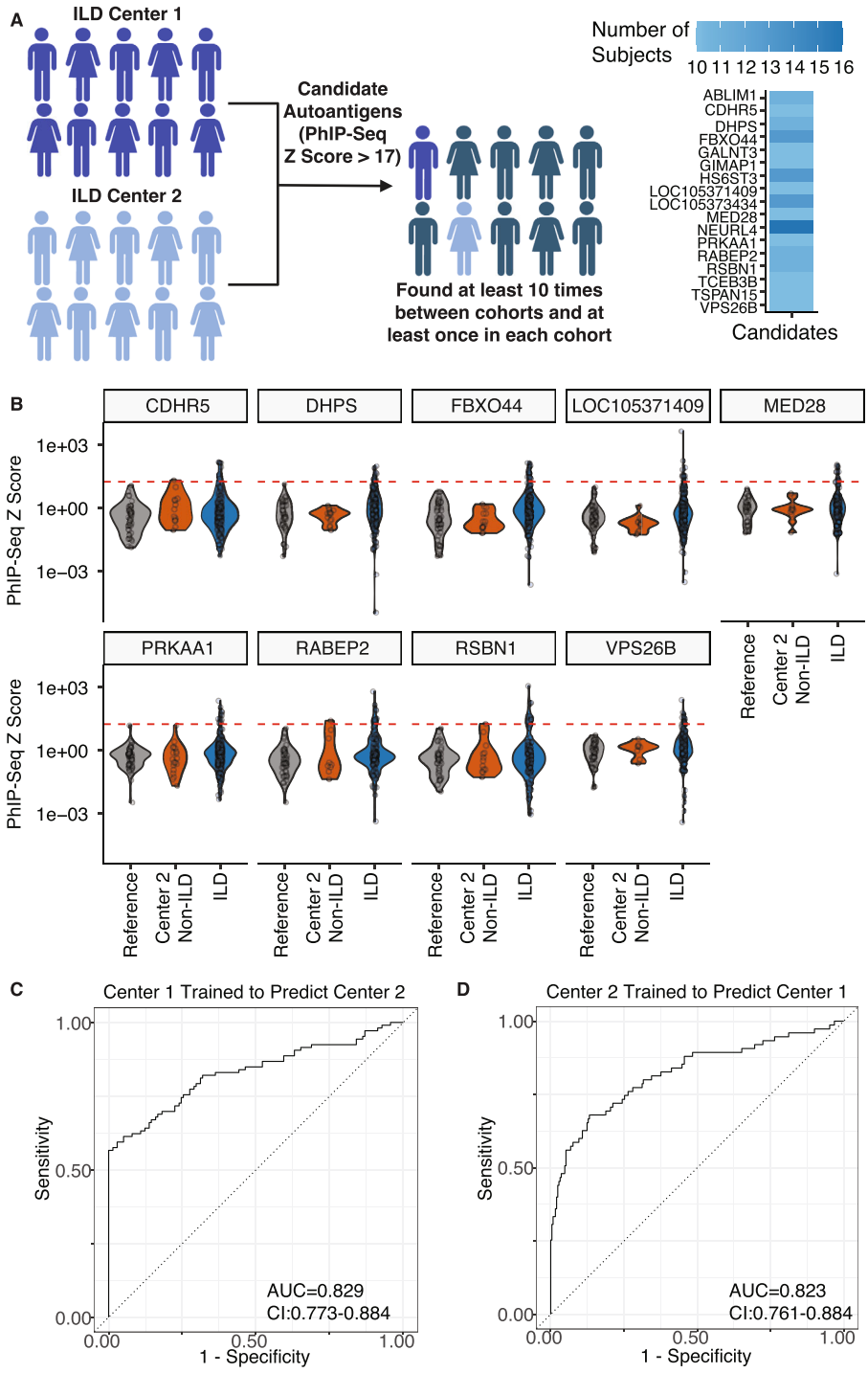

Figure 1. PhIP-Seq identifies novel candidate autoantigens with predictive capacity for ILD. (A) Candidate autoantigens were selected by identifying genes with PhIP-Seq Z scores greater than 17 in each cohort and excluding genes in the blood bank control samples that had any Z scores greater than 17. Candidates were further filtered as being found 10 times between both cohorts and found at least once in each cohort. A heatmap showing the number of individual patients for each candidate is shown for all 17 candidates. (B) Candidate autoantigens selected in (A) with limited reactivity in the Center 2 non-ILD group. The screened population including numbers of patients and samples described in Table 1 for panels A-B. (C-D) A random forest classifier was trained on PhIP-Seq data derived from all screened patients and trained either on (C) Center 1 to predict Center 2 or (D) vice versa to distinguish between patients with and without ILD. Patients with known RA without known ILD were excluded from (C-D) given potential overlap of autoreactivity with patients with RA and ILD. AUC and 95% CI are annotated on the graphs. A total of 181 samples from 169 patients with non-ILD and 388 patients with known ILD excluding 10 individuals with ILA in (C-D). 95% CI, 95% confidence interval; AUC, area under the curve; ILD, interstitial lung disease; PhIP-Seq, Phage Immunoprecipitation-Sequencing; RA, rheumatoid arthritis.

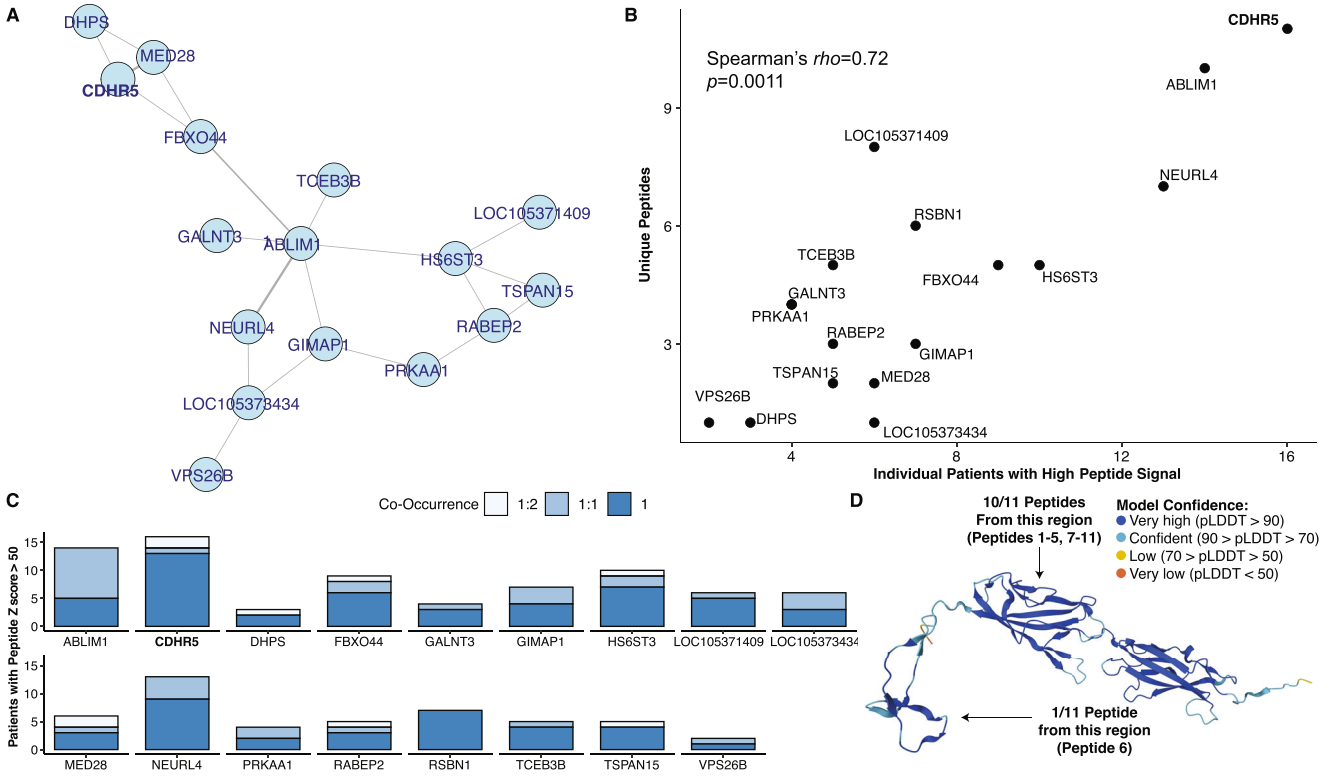

Figure 2. CDHR5 is a unique candidate autoantigen in a subset of patients with ILD. (A) Network of interactions for peptide data using a Z score threshold of 50 per individual patient. Each edge indicates co-occurrence of given candidates within screened patients. Each node represents a gene for which peptides were detected. (B) Number of unique peptides for a given autoantigen is plotted against the number of unique patients with peptide autoreactivity. A Spearman's correlation coefficient and *P* value is annotated. (C) Co-occurrence of both gene and peptide data by total number of positive patients with darker intensity colors indicating uniqueness (1:2 indicates the designated autoantigen was found with two other autoantigen candidates in a given patient; 1:1 indicates the designated autoantigen was found with one other autoantigen candidates in a given patient; 1 indicates the specified autoantigen was found in the absence of the remaining 16 candidate autoantigens). (D) Predicted structure of CDHR5 protein using AlphaFold with UniProt accession A0A7L0FKT8; per-residue confidence score (pLDDT) indicated in legend inset. Sites of CDRH5 peptides from Supplementary Table 2 annotated. ILD, interstitial lung disease; pLDDT, predicted local distance difference test.

Validation by RLBA, RNA sequencing, and immunohistochemistry. RLBA was used to validate CDHR5 by translating 35S-labeled CDHR5 using the rabbit reticulocyte lysate system and quantifying the immunoprecipitation with patient serum as described previously.[24] The CDHR5 vector was prepared by cloning the full-length copy DNA sequence (XM_011520188) led by the Kozak sequence (GCCACC) into the pTNT vector (L5610, Promega). Commercial anti-CDHR5 (PA5-89483, Invitrogen) was used as the positive control. CDHR5 positivity was defined as three SDs above the mean of the control patients as described in the text. A total of 699 samples were tested via RLBA with insufficient serum available from 5 of the original samples screened by PhIP-Seq.

Lung tissue from biopsy specimens for two CDHR5 autoreactive patients were available for sequencing and one patient for immunohistochemistry. Samples underwent RNA extraction and were subjected to bulk RNA sequencing as described previously using three age matched unused donor lung tissue samples.[25] These same patients were used for immunohistochemistry for CDHR5 using a commercially purchased antibody (HPA009081, Sigma Aldrich), and the findings discussed in the text were described by a trained pathologist (KDJ). For high resolution images, images from one donor are paired with the single CDHR5 autoreactive patient.

Survival analysis. Data on survival were conducted using the survminer package from R (survival 3.5–7, survminer 0.4.9). For the outcome of death, the years from baseline ILD clinic visit to the date of death were used for the survival analysis. For the composite outcome of death or lung transplantation, the date of death or the date of lung transplantation was used, and the data are presented with respect to years from baseline ILD clinic visit. Where death occurred, a value of 2 was provided to indicate death, and where patients were known to be alive, a value of 1 was used indicating censored data. Survival data for one screened patient (SF001310) were unavailable, and these data were filled as not applicable for the purposes of the analysis

software. Log-rank *P* values were outputted from the survminer software and annotated on the figures.

RESULTS

We selected patients from two academic medical centers with established ILD programs and experience in the multidisciplinary diagnosis of ILD. Because our goal was to discover novel autoantibodies irrespective of whether a defined CTD had been established in a patient, we started with a heterogenous group of patients. To control for variability in diagnostic agreement between the multidisciplinary diagnosis of the institutions, we specifically looked for autoantibodies shared between centers (see Methods and Supplementary Table 1A-B).

We screened 398 patients with ILD and 138 individuals whose serum was obtained from blood banks that formed our reference cohort for autoantigen candidate selection.[17] We included an additional group of patients with RA without known ILD (n = 15) and patients who were relatives of patients with ILD without known ILD (n = 16) or organ donors from Center 2 (n = 15) whose lungs were not used for transplantation; because these latter samples all had some clear biologic feature that makes them distinct from the blood donors who were used as the reference population, we include them as a separate group of patients recruited to Center 2 but without ILD (Center 2 non-ILD). Including technical replicates, we completed PhIP-Seq on 704 total samples from 582 patients (Table 1). Screened patients represented a broad array of diagnostic categories (Table 1), which reflected major diagnoses commonly referred to the ILD centers included in this study. We studied similar numbers of male (48.1%) and female (49.8%) participants. Although the majority of patients with ILD were White (69.1%), our screen included representation from multiple distinct demographic groups assessed by self-identified race and ethnicity categories (Table 1).

We used PhIP-Seq to select candidate autoantigens by using stringent Z score cutoffs for autoreactive targets and focused on targets found at least once in both ILD programs (see Methods), reasoning these would be most likely to validate in orthogonal assays and be clinically meaningful (Figure 1A). Using this approach, we identified 17 candidate autoantigens shared between ILD programs (Figure 1A-B and Supplementary Figure 1A). All reactive peptide epitopes for these 17 targets in patients with ILD are listed along with the screened patients in which they were identified (Supplementary Table 1C). Most of these candidate autoantigens were found 10 times between cohorts, though some were found more frequently (range 10–16, Figure 1A heatmap). Nine of these 17 had little to no reactivity in the Center 2 non-ILD group (Figure 1B) with the remaining 8 having similar reactivity between patients with ILD and the Center 2 non-ILD group (Supplementary Figure 1A). We compared our data to autoantibodies previously reported in the ILD literature (Supplementary Figure 1B). PhIP-Seq detected a subset of these, though none of the previously described autoantibodies had similar PhIP-Seq reactivity to those we identified in this study.

A close examination of our candidates revealed that many but not all the autoantigens are derived from proteins expressed within lung tissue, and the proteins have a variety of molecular functions or localize to a variety of cellular compartments.[26–29] The candidate autoantigens we discovered were diverse and without easily discernible relationships to one another, aside from their PhIP-Seq derived autoreactivity in patients with ILD. The number of patients demonstrating autoreactivity was approximately 2.5% of the total cohort and included patients from all the groups represented in the screen (Figure 1B and Supplementary Figure 1A). This level of autoreactivity has been reported for other autoantigens[30] and reflected that putative autoreactivity as measured by PhIP-Seq was inline with prior studies on autoreactivity in ILD. Because the patients and autoantibodies were heterogeneous, we performed a computational analysis of the data using machine learning to uncover complex associations not otherwise apparent after initial review.

The Z score data used for candidate selection were distributed over a range of values in all patients, including those patients not reaching the threshold for candidate selection. Using the full range of data, a logistic regression derived machine learning algorithm has previously been used to discriminate patients from controls using PhIP-Seq input data with respect to a hereditary form of autoimmunity (eg, APS1[17]). Here, we trained a random forest classifier to test how PhIP-Seq derived candidate autoantigens would perform as a serum-based assay to assess for the presence of ILD. We trained our classifier using PhIP-Seq data for the 17 candidates in which the classifier was trained on the data from one center and tested on the other, or vice versa (Figure 1C-D). Control samples included the individuals without ILD group (n = 138 patients, samples from the group designated Center 2 non-ILD without RA n = 31 patients, for a total of 181 control samples). There was no overlap between the training and test data sets for each classifier. We used samples from patients with ILD excluding patients with ILA because this categorization was only used at Center 1 resulting in a total of 388 patients with ILD. We found PhIP-Seq derived autoantibodies maintained the capacity to distinguish between ILD and control samples even when the center of origin was distinct (area under curve 0.829, CI 0.773–0.884; and area under curve 0.823, CI 0.761–0.884) (Figure 1C-D). These data show PhIP-Seq uncovered a panel of candidate autoantigens that are both novel and unique to patients with ILD compared with a non-ILD reference population.

We next examined whether any of the autoantibodies could define a specific subset of patients with ILD. First, we built a network diagram of interactions to indicate how frequently an autoantigen candidate was found in association with other candidates across samples (Figure 2A). All the autoantigen candidates in our panel occurred in association with at least one other and

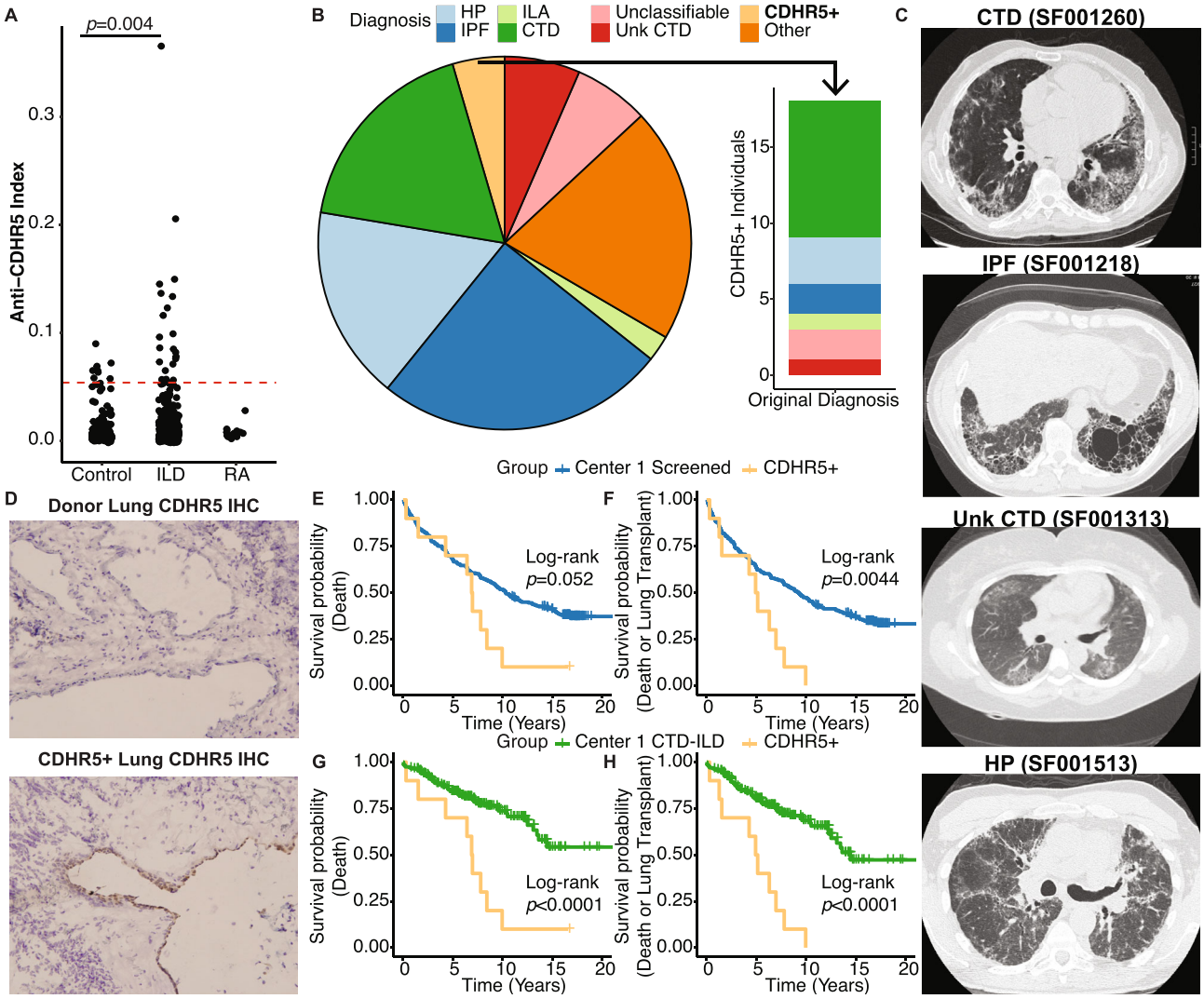

Figure 3. CDHR5 autoantibodies identify patients with progressive ILD. (A) RLBA results for CDHR5 autoreactivity (anti-CDHR5 index on y-axis) plotted by groups. Patients are separated by having RA without known ILD (RA), all other non-ILD samples as Control, and all patients with ILD in the ILD group (n = 223 Control samples, n = 461 ILD samples, n = 15 samples for patients with RA without known ILD). Wilcoxon test *P* value annotated for $P < 0.05$. (B) Patients with autoreactive CDHR5 indicated by the light orange wedge in the pie chart (CDHR5+, left), and the breakdown of these patients based on their original diagnoses is shown in the bar chart (right). (C) Representative images of computed tomography scans from four disparate ILD categories for the patients with CDHR5+. (D) CDHR5 immunohistochemistry from unused donor lung and a patient with CDHR5 Ab+ lung tissue. (E-H) Kaplan-Meier curves for survival probability accounting for the outcome of death (E, G) or accounting for the outcomes of death and lung transplantation (F, H). In (E-H) survival data from 10 patients with CDHR5+ from Center 1 are included; in (E-F) survival data from 250 patients from Center 1 included in this study; and (G-H) includes an additional 195 patients with CTD ILD from Center 1. A log-rank *P* value is annotated in (E-H). CTD, connective tissue disease; HP, hypersensitivity pneumonitis; ILA, interstitial lung abnormality; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; RA, rheumatoid arthritis; RLBA, radioligand binding assay.

none were found in isolation. When we used the specific peptide epitopes for our analysis (which are filtered to Z score cutoffs of 50), we found CDHR5 had the most unique profile (Figure 2B-C). All but one of the 11 unique CDHR5 peptides from patients with ILD comprised overlapping amino acid sequences that mapped to amino acids 155-271 of the protein (Supplementary Table 2). Further analysis of the predicted CDHR5 secondary structure revealed that a putative β-pleated sheet predicted to be an internal domain of the protein is potentially the immunodominant epitope targeted by 10 out of 11 autoantibodies (Figure 2D).

Patients showed varying levels of reactivity to CDHR5 based on PhIP-Seq Z scores (Supplementary Figure 2). Participants with hypersensitivity pneumonitis (HP) had significantly higher Z scores than those with idiopathic pulmonary fibrosis (IPF) or other forms of ILD, and patients with CTD ILD showed *P* values that exhibited trends in the same direction (Supplementary Figure 2). None of the other candidate autoantibodies exhibited discriminatory behavior by diagnoses when analyzed similarly (Supplementary Figure 2, Kruskal-Wallis $P > 0.05$). For patients with CDHR5 autoreactivity, these data suggest a clinical syndrome like CTD ILD or

Table 2. Clinical data from chart review of patients with CDHR5 autoreactivity validated by radioligand binding assay*

ID	Age	Self-identified race and ethnicity	Diagnosis	Radiographic data	Pathologic data	Serologic data	Treatment
SF000930	82	Unknown	ILA	Nonspecific interstitial markings			Antibiotics (Initial therapy)
SF001092	42	Black	CTD ILD (Scleroderma)	Bilateral basilar predominant traction bronchiectasis. Scattered ground glass		anti-Scl-70 positive (normal SSA/SSB)	Immunosuppression (Cytoxan)
SF001313	37	Black	Unk CTD	Fibrotic nonspecific interstitial pneumonia	Fibrosing and cellular nonspecific interstitial pneumonia	ANA 1:640, speckled (normal SM, RNP, SSA, SSB, anti-Scl-70, RF)	Immunosuppression (Cytoxan and prednisone)
SF001513	67	White	HP	Upper lobe predominant traction bronchiectasis. No evidence of air trapping.	Diffuse alveolar septal thickening with poorly formed granulomas	None (normal ANA, anti-Scl-70, RF)	Immunosuppression (Mycophenolate and prednisone)
SF002014	76	Asian	IPF	Basilar predominant honeycombing consistent with usual interstitial pneumonia pattern		RF 20 (normal cANCA, pANCA, anti-PR3, anti-MPO, ANA, CK)	Antifibrotic (Nintedanib)
SF002021	48	White	HP	Centrilobular nodules and ground glass opacity associated with air trapping	Cellular interstitial pneumonia with nonnecrotizing granulomas consistent with hypersensitivity pneumonia	(normal Scl-70, SSA, SSB, SM, RNP, ANA, RF, CCP, anit-PR3, anti-MPO)	Immunosuppression (Mycophenolate and prednisone)
SF002094	61	White	Unclassifiable ILD				
SF001218	59	White	IPF	Reticulation, traction bronchiectasis in a predominantly peripheral subpleural pattern suggestive of fibrotic nonspecific interstitial pneumonia	Usual interstitial pneumonia pattern	(normal ANA, antimitochondrial antibody)	Treatment of gastroesophageal reflux disease
SF001255	78	White	Unclassifiable ILD	Bilateral basial reticulation with traction bronchiectasis		(normal ANA, RF)	Treatment of gastroesophageal reflux disease
SF001260	70	White	CTD ILD (MCTD)	Bibasilar and peripheral predominant traction bronchiectasis, likely usual interstitial pneumonia pattern	Usual interstitial pneumonia with increased bronchiolocentric fibrosis	RNP 56.8 (normal ANA, dsDNA, SM, RNP, SSA, SSB, Scl-70, CCP, Pm-Scl, RNA Polymerase III, RF, C3, C4)	Immunosuppression (Mycophenolate)
case_055	42	Black	CTD ILD (Antisynthetase)	Nonspecific interstitial pneumonia		PL-12 weak positive, +SSA, +SSB, (normal ANA, dsDNA, ANCA, RF, CCP, C3, C4, anti-Jo1)	Immunosuppression (Prednisone; Rituximab, cyclophosphamide, azathioprine, hydroxychloroquine) and antifibrotic (nintedanib)

(Continued)

Table 2. (*Cont'd*)

ID	Age	Self-identified race and ethnicity	Diagnosis	Radiographic data	Pathologic data	Serologic data	Treatment
case_003	52	Black	CTD ILD (MCTD)	Usual interstitial pneumonia		ANA, Smith/RNP, RF, CCP, dsDNA (normal Scl-70, SSA, SSB, Jo-1)	Immunosuppression (Mycophenolate and prednisone)
case_120	64	White	CTD ILD (MCTD)	Probable usual interstitial pneumonia pattern peripheral predominant fibrosis	Usual interstitial pneumonia fibrosis	pANCA, RNP (normal ANA, dsDNA, SSA, SSB, Smith)	Immunosuppression (Azathioprine and prednisone)
case_012	79	Black	CTD ILD (MCTD)	Usual interstitial pneumonia pattern with overlying emphysema		ANA, RF, CCP, (normal dsDNA, C3, C4)	Immunosuppression (Leflunomide and prednisone)
case_042	75	White	CTD ILD (RA)	Possible usual interstitial pneumonia pattern subpleural reticulation and mosaicism also noted	Fibroblastic foci, focal areas of nonspecific interstitial pneumonia, though overall a usual interstitial pneumonia pattern	ANA, ANCA, (normal RF and CCP)	Immunosuppression (Prednisone, mycophenolate, hydroxychloroquine)
case_066	53	Unknown	CTD ILD (RA)	Upper lobe predominant fibrosis, air trapping nonspecific interstitial pneumonia vs HP pattern	Combined nonspecific interstitial pneumonia and fibroblastic foci, honeycombing, germinal centers, lymphoid aggregates, rare giant cells	ANA, RF, CCP (normal SSA, SSB)	Immunosuppression (Prednisone, azathioprine, rituximab)
case_086	69	Black	CTD ILD (MCTD)	Usual interstitial pneumonia pattern with exuberant honeycombing		ANA, SSA, Sm, RNP, dsDNA, pANCA (normal Jo-1, Scl-70, RF, SSB)	Immunosuppression (Prednisone, mycophenolate, hydroxychloroquine)
case_020	68	White	HP	Mosaic attenuation		Pos ANA (normal Scl 70, dsDNA, ANCA, SSA, SSB, Sm, RNP)	Immunosuppression (Azathioprine and prednisone)

*The patient identifier is indicated by the column labeled ID. Age at study enrollment is listed for Age. The original diagnosis by multidisciplinary conference is listed in the column labeled Diagnosis. In the column listed Treatment, the type of treatment is specified in parentheses where relevant. Where data were not available after extensive chart review, values are left blank.

ANA, antinuclear antigen antibody; anti-Jo1, antihistidyl-transfer RNA synthetase antibody; anti-Scl-70, antitopoisomerase I antibody; C3, complement component 3; C4, complement component 4; cANCA, cytoplasmic antineutrophil cytoplasmic antibody; CCP, anticyclic citrullinated peptide; CK, creatinine kinase; CTD ILD, connective tissue disease interstitial lung disease; dsDNA, double stranded DNA; HP, hypersensitivity pneumonitis; ILA, interstitial lung abnormality; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; MCTD, mixed connective tissue disease; MPO, myeloperoxidase; pANCA, perinuclear antineutrophil cytoplasmic antibody; PL-12, antialanyl-tRNA synthetase antibody; PM-Scl, antiexosome antibody; PR3, proteinase 3; RNP, antinuclear ribonuclear protein antibody; RA, rheumatoid arthritis; RF, rheumatoid factor; SSA, anti-Ro antibody; SSB, anti-La antibody; SM, smooth muscle antibody; Unk CTD, unknown connective tissue disease.

HP may be identifiable (Supplementary Figure 2 and Table 2). Taken together, CDHR5 appeared to be an autoantibody candidate with strong potential to define a novel subtype of ILD. We therefore sought to validate this further with an orthogonal experimental approach.

To validate the presence of CDHR5 autoantibodies in patients, we developed a CDHR5 RLBA and tested the serum from all screened patients and controls. By applying this test to 699 samples, we confirmed the presence of CDHR5 autoantibodies in 18 patients with ILD (Figure 3A). CDHR5 autoantibody titers were elevated in ILD samples compared with control samples (Figure 3A). Patients with autoantibody-positive CDHR5 were characterized by several ILD categorizations (Figure 3B and Table 2). Half of the patients (9/18) had prior evidence of autoimmunity and a defined systemic rheumatic disease including mixed CTD (n = 4), RA (n = 2), antisynthetase syndrome (n = 1), and scleroderma (n = 1). Importantly, CDHR5 autoantibodies were detected in 9 of 18 patients without known autoimmunity, including one patient who met criteria for idiopathic pneumonia with autoimmune features and two patients with unclassifiable ILD. Other diagnoses included HP (n = 3), IPF (n = 2), and ILA (n = 1) (Table 2).

We performed detailed chart reviews of patients with CDHR5 autoantibodies and analyzed clinical, radiographic, and pathologic data (Table 2 and Figure 3C). For all patients classified as HP, a culprit antigen for inciting the disease was never identified and the diagnosis was not considered definitive for any patient.[31] Although patients with autoreactive CDHR5 with known CTD were uniformly given immunosuppressive therapy, patients without a known CTD had more variable treatments, including antifibrotic medications or treatment of gastroesophageal reflux disease (Table 2). The mean age of patients with a CDHR5 autoantibody was 62.3 ± 13.9 years (mean ± SD), which overlapped with all subgroups of screened patients (Table 1).

Although CDHR5 is not known to be expressed in respiratory tissue, we conducted an exploratory analysis to assess whether we could detect CDHR5 protein in the lungs of patients with confirmed CDHR5 autoreactivity. We located lung tissue amenable to histology from one patient with CDHR5 autoantibodies validated by RLBA and conducted immunohistochemistry using a commercially available antibody against CDHR5. CDHR5 staining was strongly detected in the respiratory bronchial epithelium the patients with autoreactive CDHR5 (n = 1 out of 1 patient), but notably not in the lungs of control patients (n = 1 out of 3 a patient with a low degree of CDHR5 autoreactivity) (Figure 3D). We saw no difference in this staining pattern when looking at areas of lung affected by fibrosis (Supplementary Figure 3 and Supplementary Image Folder 1). We performed bulk RNA sequencing of banked lung tissue available from two patients with autoreactive CDHR5 and compared these results with data derived from age matched normal lung tissue (n = 3 patients).[25] We found 2,268 genes differentially expressed between the groups (Benjamini-Hochberg adjusted $P < 0.1$, Supplementary Figure 4), including several genes linked to fibrotic lung disease.[32,33]

Because CDHR5 autoantibodies may have been identifying a subset of individuals with CTD ILD as the main cause of their pulmonary fibrosis, we wanted to understand whether patients with autoantibody-positive CDHR5 had any differences in clinical outcomes. We leveraged available data from Center 1 on the survival data of screened patients for this study. We conducted analyses using an endpoint of death and a composite endpoint of transplant-free survival defined by the date of death or lung transplant surgery, reasoning that the date of lung transplantation is comparable with death because it denotes end-stage failure of the patient's native lungs (Supplementary Table 1D). In contrast to other members of the Center 1 screened cohort, patients with CDHR5 autoantibodies had worse survival when accounting for death and lung transplantation (Figure 3E-F). When compared with historical survival data of patients with CTD ILD from Center 1, patients with autoantibody-positive CDHR5 were also found to have worse survival (Figure 3G-H). In addition, five of the eight patients with CDHR5 autoreactivity at Center 2 were deceased within 10 years of initial visit, and one of the surviving patients underwent lung transplantation within a year of their initial ILD visit. Taken together, our study suggests that patients with CDHR5 autoantibodies represent a unique subset of patients with ILD with progressive lung disease.

DISCUSSION

In this study, we used PhIP-Seq to perform a large unbiased autoantibody discovery screen in ILD drawing from two separate ILD centers. Because the diagnosis of ILD is challenging with potential for misclassification, we studied a heterogeneous cohort of patients, irrespective of whether patients had a confirmed (or suspected) systemic rheumatic disorder. We identified 17 novel candidate autoantigens associated with ILD, none of which have been previously described. We validated CDHR5 autoantibodies in 18 patients with ILD. Autoantibodies to CDHR5 were found in patients with defined autoimmune disorders providing a direct link to conditions of impaired immunologic tolerance in this patient population. We also discovered CDHR5 autoantibodies in patients without an established autoimmune disease. PhIP-Seq enables groups to identify and then subsequently validate autoantibodies that serve as diagnostic tools and useful additions to a multidisciplinary ILD evaluation, including one performed at an established ILD program. Our data also add to prior evidence that standard serologic tests ordered during a routine ILD evaluation are insufficient to capture unique subtypes of autoimmune-associated ILD, particularly those that are distinct from established rheumatologic diseases.

Importantly, in COVID-19[12–14] and other diseases,[10,11] autoantibodies can have a key role in disease pathogenesis, even in the absence of a systemic rheumatic disease. Autoantibodies to

type 1 interferons[12–14] or surfactant proteins[11] can worsen lung damage either through their direct suppression of immune responses or by the physiologic derangements they provoke in the lung. Although the precise mechanisms by which these types of autoantibodies arise are not well understood, these studies highlight the importance of autoantibody discovery in patients not typically suspected of harboring pathogenic autoantibodies. Although we have not yet shown that CDHR5 autoantibodies mediate fibrosis or inflammation, we validated it as a novel autoantigen and discovered autoreactivity to 16 other potential autoantigens in patients with ILD.

The diagnosis and classification of ILD is challenging, and the practice at specialized centers involves a multidisciplinary discussion of each patient integrating clinical, pathologic, and radiographic information by experts.[34] Because diagnostic agreement between institutions can vary,[35] objective measures can minimize misclassification of ILD. Specific autoantibodies fill this need by enabling clinicians to diagnose subtypes of ILD that provide insight into treatment and prognosis (eg, Melanoma Differentiation-Associated gene 5 [MDA-5] autoantibody-positive dermatomyositis ILD[36,37]) or mechanistic links to certain defects in immune tolerance.[24,38] The in-depth analysis of CDHR5 autoantibodies we performed in this study hints at the potential of PhIP-Seq to uncover a shared antibody in seemingly disparate patients. To place anti-CDHR5 antibody positive ILD into the broader context of autoimmune ILD, we performed electronic query of the entire UCSF ILD cohort (n = 4,484 patients) and found that 4.5% (n = 203 patients) had a diagnosis of "myositis," "antisynthetase," or "MDA5+" ILD (tabular data available on request). The frequency of these diseases in the UCSF ILD database is on par with that of our own estimate for CDHR5 autoreactivity seen in the patients from this study. Although CDHR5 autoantibody prevalence was similar to the control group, CDHR5 autoantibody titers were elevated in patients with ILD (Figure 3A). On a population level, ILD is rare and for subsets of rare diseases, true prevalence estimates are quite challenging to obtain.[39] Furthermore, analogous to antinuclear antibodies, which are long established for the diagnosis of select rheumatologic conditions, positive threshold values for CDHR5 autoantibodies will require calibration to optimize sensitivity and specificity.[40] Importantly, patients with CDHR5 autoantibodies exhibited worse mortality compared with patients with CTD ILD and other screened patients (Figure 3E-H). Although not as rapidly progressive as MDA-5 associated ILD, CDHR5 may be an analogous marker of patients with progressive ILD.

A prior gene ontology analysis revealed extensive association between CDHR5 and genes involved in transmembrane localization or intracellular transport.[41] Furthermore, CDHR5 is appreciated to be expressed within the intestines and forms a macromolecular complex with the harmonin protein in which it is required for microvilli formation.[42] If CDHR5 protein does localize similarly to harmonin, it would favor a hypothesis in which cell surface–derived CDHR5 may be an antigen that stimulates an autoantibody response. Antibodies to harmonin have been reported in immune dysregulation polyendocrinopathy enteropathy X-linked syndrome.[43] Although mutations in harmonin are causatively linked to a heritable form of deafness and blindness known as Usher syndrome,[44] our chart review revealed no similarities between the patients with major Usher syndrome phenotypes and the clinical findings seen in patients with autoantibodies to CDHR5. A recent report described bronchiectasis in Usher syndrome,[45] though the described radiographic abnormalities are distinct from traction bronchiectasis described in patients with autoreactive CDHR5 (Table 2).

We wanted to understand what cell types in ILD express CDHR5. We used the IPF Cell Atlas[46] to help inform this question. In general, CDHR5 expression was sparse, and where it was detectable, CDHR5 was found in ciliated cells,[47–49] alveolar type 2 cells,[48,50] and in potentially even in some hematopoietic cells.[49,51] Although it is notable that CDHR5 is seen in ciliated cells in these datasets, which is consistent with our histologic findings (Figure 3D), these datasets may not be ideal as they focused on patients with IPF, and our own data suggests CDHR5 is a marker of CTD ILD.

There was a recent genome wide–association study that revealed an additional 13 new risk loci for the development of systemic sclerosis.[52] Notably, the CDHR5-IRF7 gene locus was strongly linked to anticentromere antibody titer.[52] In our own study, although numerous patients had had abnormally elevated antinuclear antibody levels, we did not have data on anticentromere antibodies to correlate with CDHR5 autoantibodies. In the context of this prior study, our own data add to a growing body of evidence implicating CDHR5 in overlap autoimmune syndromes. As additional studies interrogate the molecular function of CDHR5,[41,42] it will be important to pair genetic mouse models modifying CDHR5 or IRF7 to one of several inducible models of systemic sclerosis that also cause fibrotic lung disease.[53]

Our study has several limitations. PhIP-Seq identifies epitopes encoded in linear stretches of DNA and is not designed to capture posttranslational modifications, which have a role in autoimmune syndromes.[54] In addition, we had limitations in available human lung tissue. Although we could validate CDHR5 via RLBA in 18 patients, we only had lung tissue available from two patients for transcriptional characterization and for one patient for immunohistochemistry. Finally, although we conducted detailed chart reviews, we were restricted in our ability to define additional autoantibodies that can be evaluated using clinical tests in patients with autoreactive CDHR5 because this information was not available in the electronic medical record.

Our work shows how an unbiased approach in patient selection and screening technology can identify novel autoantibodies outside the framework of currently established rheumatologic diseases. Future studies will be needed to determine whether the autoantibodies discovered here can help with improved

identification, molecular understanding, and even treatment of patients with previously poorly understood subsets of ILD.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs Shum and Sperling had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Upadhyay, Yoon, Law, Wolters, DeRisi, Sperling, Shum.

Acquisition of data. Upadhyay, Yoon, Vazquez, Velez, Jones, C. Lee, Law, S. Lee, Yang, DeRisi, Sperling, Shum.

Analysis and interpretation of data. Upadhyay, Yoon, Vazquez, Velez, Jones, C. Lee, Law, Wolters, S. Lee, Farrand, Noth, Strek, Anderson, DeRisi, Sperling, Shum.

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