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Clinical and molecular features of rapidly progressive chronic hypersensitivity pneumonitis

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Abstract. *Background:* Chronic hypersensitivity pneumonitis (CHP) is characterized by varying degrees of inflammation and fibrosis of the lungs caused by a variety of inhaled antigens. Despite extensive efforts to minimize exposure to the antigens, patients with CHP sometimes experience rapid deterioration of their pulmonary functions, resulting in death within a few years. *Objectives:* This study aimed to define clearly the clinical and molecular features of patients with rapidly progressive CHP. *Methods:* Annual decline in pulmonary functions and its association with clinical variables was evaluated in 43 patients with CHP. The RNA from frozen lung specimens of nine patients with rapidly progressive CHP and normal control subjects was profiled using Illumina HumanWG-6 v3 Expression BeadChips, and an Ingenuity Pathway Analysis was performed to identify the altered functional and canonical signaling pathways. *Results:* Patients with more than 10% annual decline in forced vital capacity and those with more than 15% annual decline in diffusion capacity for carbon monoxide showed significantly poor overall survival rates (p=0.002 and p=0.001, respectively). According to the gene expression analysis, 160 genes, including cystatin SN *(CST1)*, ephrin-A2 *(EFNA2)*, and wingless-type MMTV integration site family, member 7B *(WNT7B)* were upregulated, and pathways related to inflammatory responses and autoimmune diseases were differentially expressed. *Conclusion:* Greater annual decline in pulmonary function can predict poorer prognosis of patients with CHP. Genes and pathways related to inflammatory responses and autoimmune diseases have potential roles in the pathogenesis of rapidly progressive CHP, suggesting their potential as diagnostic biomarkers and/or therapeutic targets. *(Sarcoidosis Vasc Diffuse Lung Dis 2017; 34: 48-57)*

Key words: autoimmune diseases, biomarkers, gene expression, hypersensitivity pneumonitis, interstitial lung disease

Introduction

Chronic hypersensitivity pneumonitis (CHP) is characterized by varying degrees of inflammation and progressive fibrosis of the lungs caused by persistent exposure to a variety of inhaled antigens, including fungi, animal or bacterial proteins, and low-molecular-weight chemical compounds (1-3). Identification of the causative antigen and efforts to avoid or minimize exposure to it are key actions in the management of CHP. Pharmacological therapeutics,

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including corticosteroids, have limited efficacy in patients with CHP (3), and patients sometimes experience rapid deterioration of pulmonary function, resulting in death within a few years (4-8). Moreover, patients with usual interstitial pneumonia (UIP)-like or fibrotic non-specific interstitial pneumonia (f-NSIP)-like pattern on surgically resected lung tissue have shown a clinical outcome similar to that of patients with idiopathic pulmonary fibrosis (IPF) (4, 5, 7). Since surgical biopsy is a highly invasive procedure, it is not usually tolerable in all patients. Thus, a less invasive clinical biomarker for predicting the outcome of patients with CHP as well as a novel effective therapeutic agent is urgently required.

Serial changes in forced vital capacity (FVC) and diffusion capacity for carbon monoxide (DLco) have been reported to predict the outcome of patients with IPF in several previous studies (9-14). Because IPF and CHP have significant clinical, radiological, and pathological overlaps (15, 16), we hypothesized that annual declines in FVC and DLco can predict the functional deterioration as well as prognosis of patients with CHP.

Genome-wide microarray analysis enabled us to obtain comprehensive gene expression profiles related to detailed phenotypic and biological information for several diseases (17). This approach is also useful to identify unknown molecules in the pathways involved in various types of pulmonary fibrosis (18-27). However, few such analyses have been performed for CHP (19).

This study aimed to define the clinical and molecular features of patients with CHP and to identify potential therapeutic target molecules, especially in cases of rapidly progressive CHP with poor prognosis. First, we investigated the association between the annual decline in pulmonary function and the prognosis of patients with CHP. Second, we identified the genes and pathways that are differentially expressed in patients with rapidly progressive CHP as compared to the controls.

MATERIAL AND METHODS

Study subjects

Between November 2001 and June 2012, 43 patients with newly diagnosed CHP at Hiroshima University Hospital (Hiroshima, Japan) were enrolled in this study. Diagnoses of CHP were made according to the criteria proposed by Yoshizawa et al (1, 28, 29). In brief, the diagnostic criteria requires that three or more of the following conditions (including either i) or ii), either iii) or iv), and either v) or vi)) should be met: i) reproduction of symptoms by environmental provocation or inhalation of the antigen, ii) antibodies and/or lymphocyte proliferation targeting the specific antigen, iii) evidence of pulmonary fibrosis with or without granulomas on histopathological analysis, iv) honeycombing on computed tomography (CT), v) progressive deterioration of a restrictive impairment of pulmonary function over 1 year, and vi) persistence of respiratory symptoms related to the disease for more than 6 months. This study was approved by the Ethics Committee of Hiroshima University Hospital (approval numbers 326 and M33) and conducted in accordance with the ethical standards established in the Helsinki Declaration of 1975. All patients provided written informed consent to use their samples for this study.

Pulmonary function tests and bronchoalveolar lavage (BAL)

Spirometry and DLco measurements were performed by specialized technicians in accordance with the recommendations of the American Thoracic Society, as previously described (30-33). The rate of annual decline in FVC or DLco was calculated by dividing baseline FVC or DLco by the slope of regression line, although six patients missed follow-up measurements for DLco. BAL was performed under local anesthesia, by injecting 50 mL of saline thrice at the more severely affected area in the right middle lobe or lingula, as observed by high-resolution CT performed just before BAL (34).

RNA isolation and gene expression profiling

Gene expression profiles of frozen specimens derived from the central part of the lung by surgical biopsy in nine patients with CHP who showed more than 10% annual decline in FVC was analyzed by GP Biosciences Ltd. (Kanagawa, Japan). Control lung specimens consisted of total RNA from three lungs (Caucasians aged 32-61 years, without any concomitant lung disease, cause of death: sudden death) purchased from BD Biosciences Clontech (Lot Number 7080277; Palo Alto, CA, USA). RNA quality was checked using RNA6000 Nano Assay on Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The Illumina BeadArrays Human WG-6 v3 (Illumina Inc., San Diego, CA, USA) with about 48,000 transcripts was used for RNA profiling, according to the manufacturer's instructions. The Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, USA) was used to obtain biotin-labeled cRNA from 500 ng of total RNA. As a control probe, normal human lung poly (A) RNA (BD Biosciences Clontech) was amplified using the same amplification conditions. cRNA was synthesized overnight (18 h), labeled, and hybridized to the chip at 58°C overnight. Hybridized arrays were stained with streptavidin-Cy3 (PA43001; Amersham™, Buckinghamshire, UK) and scanned with an Illumina BeadArray Reader (Illumina Inc.). The scanned images were imported into BeadStudio v3 software (Illumina Inc.) for extraction, quality control, and quintile normalization. Satisfactory quality of the arrays and samples was observed in all cases.

Microarray data analysis

Cluster analysis was performed using Gene Cluster 3.0 and Java TreeView software developed by Eisen et al (35, 36). The analysis included 515 genes for which valid data were obtained in 80% of the experiments, and whose expression ratios varied by standard deviations of >2.5. Gene lists were further categorized into functional and pathway analysis, using the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA).

Statistical analysis

Data were analyzed with SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Data for individual variables from two groups were tested by the Mann-Whitney *U* test, with the level of significance set at $P<0.05$. Survival time was defined as the period from the date of initial consultation to the date of death due to any cause. Survival curves were analyzed by the Kaplan-Meier method, and the log-rank tests stratified for annual decline in FVC or DLco and baseline BALF lymphocyte count were performed. The division thresholds were set at annual declines of 10% and 15% for FVC and DLco, respectively, which were previously reported to be the appropriate prognostic thresholds for predicting the outcome of patients with IPF (9-13). In addition, the division threshold for BALF lymphocyte count was set at a median value of 4.0×10^4/mL. The receiver operating curves (ROC) were drawn for these three factors to confirm their ability to predict the five-year survival. The selection of covariates included in multivariate Cox proportional hazards models was based on previous studies that demonstrated the importance of gender, age, and pulmonary function as the prognostic factors for chronic interstitial lung diseases (ILDs) including CHP (37, 38).

RESULTS

Baseline characteristics

The baseline characteristics of 43 patients with CHP are presented in Table 1. There was no significant difference in age, gender, smoking status and baseline pulmonary functions between the patients with greater annual decline in pulmonary functions and those with less annual decline. On the other hand, BALF lymphocyte count was significantly higher in patients who showed less than 10% annual decline in FVC and less than 15% annual decline in DLco, as compared to those who showed greater rates of decline.

Annual decline in pulmonary function and prognosis of patients with CHP

The mean annual decline in FVC was 156.2±62.9 mL/year, which accounted for 7.7%± .1% of baseline FVC, and the mean annual decline in DLco was 0.67±0.40 mL/min/mmHg/year, which accounted for 5.6%±3.8% of baseline DLco. As shown in Figure 1, the log-rank analyses revealed that more than 10% annual decline in FVC, more than 15% annual decline in DLco, and less than 4.0×10^4/mL of BALF lymphocyte count were significant predictors of poor overall survival of patients with CHP ($P<0.001$, $P=0.001$, and $P<0.001$, respectively). ROC analysis confirmed the significant abilities of annual decline in FVC or DLco and BALF lymphocyte count to discriminate the patients who died within

Table 1. Baseline characteristics of the patients

	All	FVC decline ≥10% per year			DLco decline ≥15% per year		
		yes	no	*p* value	yes	no	*p* value
Number of the subjects	43	15	28		9	28	
Age	64.8±1.6	65.6±2.1	64.4±2.3	0.919	65.6±1.8	64.4±2.2	0.915
Gender, Male / Female	27 / 16	8 / 7	19 / 9	0.348	5 / 4	18 / 10	0.464
Smoking history, Yes / No	22 / 21	7 / 8	15 / 13	0.666	4 / 5	14 / 14	0.538
Antigen, Birds / Others	23 / 20	10 / 5	13 / 15	0.205	6 / 3	13/15	0.252
Pulmonary function test							
FVC, L	2.35±0.10	2.19±0.15	2.44±0.14	0.333	2.31±0.15	2.37±0.14	0.944
FVC, percent predicted	77.2±2.4	77.1±4.5	77.3±3.0	0.799	82.3±6.3	76.5±3.0	0.436
DLco, mL/min/mmHg	11.4±0.8	10.3±0.6	11.9±1.1	0.500	11.0±0.7	11.6±1.0	0.685
DLco, percent predicted	49.6±2.7	49.0±5.0	49.9±3.2	0.876	49.9±6.6	50.0±3.0	0.937
BALF							
Total cell count, *10^4/mL	25.0±2.2	19.0±2.0	28.2±3.0	0.083	20.6±5.1	26.7±2.9	0.202
Macrophage, *10^4/mL	15.3±1.2	14.0±1.2	16.0±1.8	0.819	15.6±3.4	14.5±1.5	0.860
Lymphocyte, *10^4/mL	7.2±1.4	2.7±0.8	9.6±2.0	0.002	2.3±0.8	9.7±2.0	0.009
Neutrophil, *10^4/mL	1.7±0.3	1.7±0.5	1.7±0.4	0.959	1.6±0.7	1.7±0.4	0.547
Eosinophil, *10^4/mL	0.8±0.2	0.7±0.3	0.9±0.3	0.919	1.1±0.6	0.8±0.3	0.750

The patients were classified according to the decline rate of each, FVC and DLco. Values are expressed as mean ± SEM or number. Abbreviations; FVC, forced vital capacity; DLco, single-breath diffusing capacity of lung for carbone monoxide; $FEV_{1.0}$ forced expiratory volume in 1 second; FVC, forced vital capacity

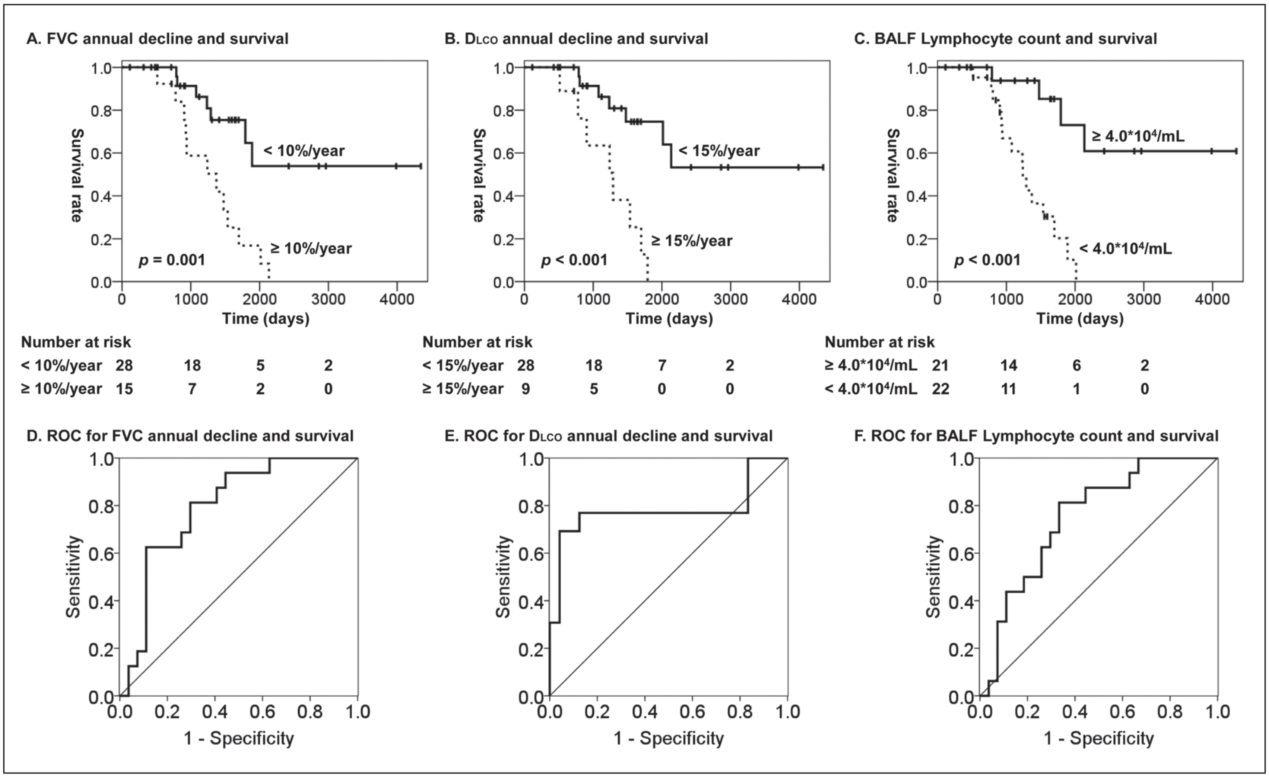

Fig. 1. Overall survival of the forty-three patients with CHP was assessed in relation to (A) annual decline in FVC, (B) annual decline in DLco and (C) BAL lymphocyte count. Differences between the two groups were evaluated using a log-rank test. Number at risk is the number of patients whose follow-up continues at each time point. ROC analysis for (D) annual decline in FVC (area under the curve (AUC) = 0.796, $p = 0.001$), (E) annual decline in DLco (AUC = 0.782, $p = 0.005$) and (F) BAL lymphocyte count (AUC = 0.752, $p = 0.006$) to discriminate the patients who die within five year from the patients who survive

five year from those who survive. Further, multivariate Cox proportional hazard analyses revealed that annual decline in both FVC and DLco, as well as the baseline BALF lymphocyte count, are significantly associated with the survival of CHP patients independently from other clinical factors including gender, age, and baseline pulmonary function (Table 2).

Cluster analysis of gene expression profiles

The clinical characteristics of the nine patients with CHP who were enrolled in the gene expression analysis are shown in Table 3. An unsupervised two-dimensional hierarchical clustering algorithm was used to analyze the similarities among the samples and genes (Figure 2). The two major groups CHP1-4 (Group 1) and CHP5-9 (Group 2) were distinguished based on their expression data. However, there were no significant differences in the clinical characteristics between the groups (Table 3).

Identification of genes upregulated in rapidly progressive CHP

In total, 160 genes were upregulated while 832 were downregulated in the lung tissues of patients with rapidly progressive CHP as compared to control subjects (expression ratio >20.0 and <0.05, respectively), in at least 75% (*i.e.*, seven out of nine) of the patients; the top 50 upregulated genes are listed in Table 4. Some of these genes, namely secreted frizzled-related protein 4 *(SFRP4)*, docking

Table 2. Multivariate Cox proportional hazard analyses in two models

	Model 1			Model 2		
Variable	HR	95% CI.	*p* value	HR	95% CI.	*p* value
BALF lymphocyte, <4.0*10^4/mL	8.61	2.38-31.20	0.001	10.95	2.21-54.34	0.003
FVC decline, ≥10%/year	8.89	2.85-27.73	<0.001	9.55	2.84-32.07	<0.001
DLco decline, ≥15%/year	24.72	4.43-138.07	<0.001	7.64	2.21-26.43	0.001

The independent associations between survival and BALF lymphocyte, FVC decline or DLco decline were tested with following covariates; Model 1 includes gender, age and baseline FVC (percent predicted), Model 2 includes gender, age and baseline DLco (percent predicted)

Table 3. Clinical characteristics of the patients for gene expression analysis

	All	Group 1	Group 2	P value
Number of the subjects	9	4	5	
Age	63.3±2.8	67.3±5.1	60.2±2.6	0.387
Gender, Male / Female	4 / 5	2 / 2	2 / 3	0.643
Smoking history, Yes / No	4 / 5	2 / 2	2 / 3	0.643
Antigen, Birds / Others	6 / 3	2 / 2	4 / 1	0.405
Pulmonary function test				
FVC, L	2.3±0.2	2.0±0.5	2.4±0.2	0.327
FVC, percent predicted	78.5±6.9	67.8±7.6	87.0±9.8	0.086
DLco, mL/min/mmHg	10.2±1.0	ND	10.6±1.2	
DLco, percent predicted	50.7±5.6	ND	62.8±4.5	
BALF				
Total cell count, *10^4/mL	19.4±2.8	23.3±3.3	16.3±3.9	0.221
Macrophage, *10^4/mL	14.6±1.7	17.6±0.8	12.1±2.6	0.142
Lymphocyte, *10^4/mL	2.6±0.8	2.6±1.1	2.5±1.2	0.806
Neutrophil, *10^4/mL	1.4±0.6	2.2±1.3	0.7±0.4	0.327
Eosinophil, *10^4/mL	0.9±0.4	1.0±0.8	0.9±0.5	0.902
Decline in pulmonary function				
FVC annual change, mL/year	481.7±65.9	529.0±144.2	443.8±48.7	0.624
FVC relative change, %/year	23.9±5.9	30.7±13.1	18.5±2.1	0.624
DLco annual change, mL/min/mmHg/year	1.7±1.0	ND	2.4±0.8	
DLco relative change, %/year	16.3±10.8	ND	24.0±9.7	

The two major groups CHP1-4 (Group 1) and CHP5-9 (Group 2) were distinguished according to the cluster analyses based on their gene expression data as shown in Figure 2. Values are expressed as mean ± SEM or number. Abbreviations; FVC, forced vital capacity; DLco, single-breath diffusing capacity of lung for carbone monoxide; $FEV_{1.0}$ forced expiratory volume in 1 second; FVC, forced vital capacity; ND, no data

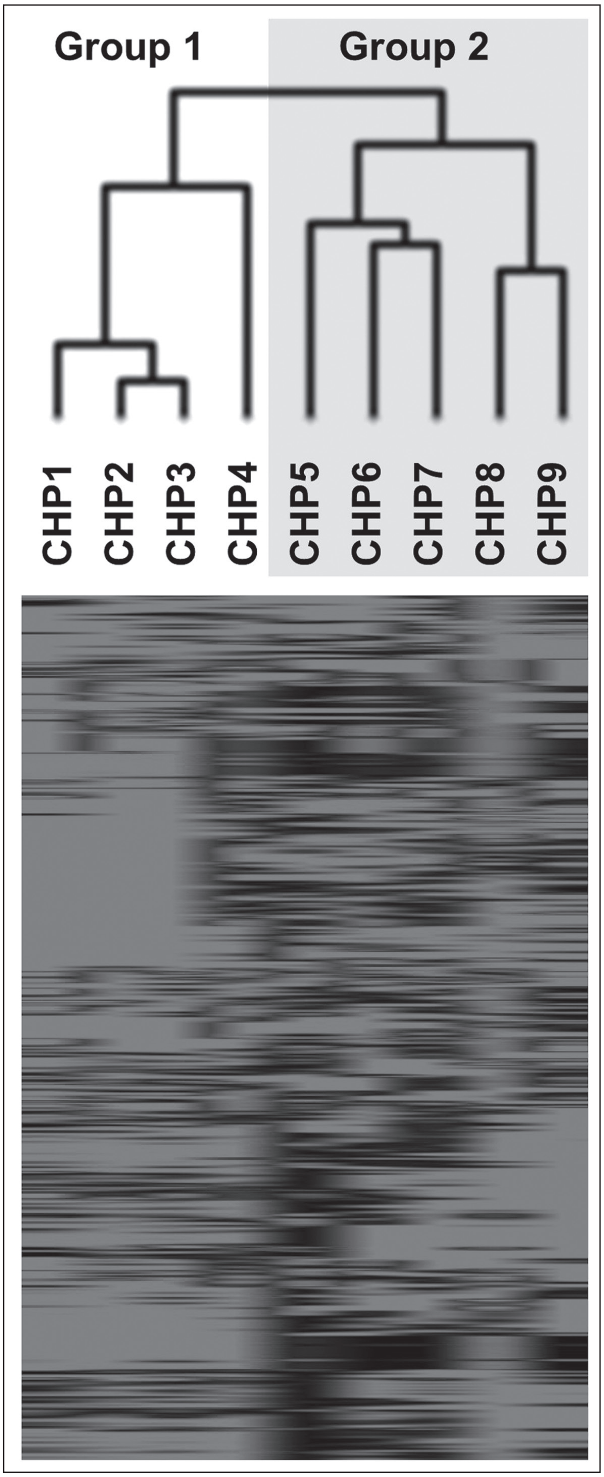

Fig. 2. Cluster analysis of gene expression profile was performed for nine patients with rapidly progressive CHP. The dendrogram (top) indicates similarities between cases, with shorter branch indicating higher similarity. Within the heatmap (bottom), red represents the upregulated gene expression and green represents the downregulated gene expression

protein 5 *(DOK5)*, wingless-type MMTV integration site family, member 7B *(WNT7B)*, are known to be involved in cell differentiation; some, namely ephrin-A2 *(EFNA2)*, protocadherin 7 *(PCDH7)*, osteomodulin *(OMD)*, are known to be involved in cell-cell interaction or cell adhesion; and the others, namely matrix metallopeptidase 11 *(MMP11)*, cystatin SN *(CST1)*, and cystatin SA *(CST2)*, are known proteases or antiproteases. Of these, WNT7B and CST1 have also been previously reported as serum biomarkers for ILDs (39, 40).

Biological pathway analysis

As shown in Table 5, IPA software estimated the differently expressed gene sets based on three categories: 1) diseases and disorders; 2) molecular and cellular functions; and 3) physiological system development and function. The top entry in each category was neurological disease, cell death and survival, and tissue development, respectively. The top five canonical signaling pathways comprised tumor necrosis factor receptor-1 (TNFR1) signaling, TNFR2 signaling, integrin signaling, triggering receptor expressed on myeloid cells-1 (TREM1) signaling, and those implicated in molecular mechanisms of cancer.

Discussion

In this study, we evaluated the clinical and molecular features of patients with CHP. Patients with CHP who showed a greater decline in FVC and/or DLco showed significantly poorer prognosis. To our knowledge, this is the first report demonstrating the independent association between changes in FVC or DLco over time and the prognosis of patients with CHP. Further, a gene expression analysis in patients with rapidly progressive CHP identified several genes that could be deeply involved in the molecular pathogenesis of CHP and could be useful diagnostic biomarkers or therapeutic targets.

Multivariate Cox proportional hazard analyses demonstrated that annual declines in FVC and DLco, as well as BALF lymphocyte count were independent prognostic factors in patients with CHP (Table 2). Vourlekis et al. previously reported that a decreased BALF lymphocyte count in patients with CHP is significantly associated with lung fibrosis as

Table 4. Top 50 upregulated genes in the rapid progressive CHPs

Gene symbol	Gene Name	Fold change patients with CHP /control
CST1	cystatin SN	308.4
C4A/C4B	complement component 4B (Chido blood group)	262.5
ARSH	arylsulfatase family, member H	201.7
BAAT	bile acid CoA: amino acid N-acyltransferase (glycine N-choloyltransferase)	172.9
ZNF675	zinc finger protein 675	171.6
ATP4B	ATPase, H+/K+ exchanging, beta polypeptide	169.9
SFRP4	secreted frizzled-related protein 4	169.5
KRTAP10-4	keratin associated protein 10-4	167.3
EFNA2	ephrin-A2	166.6
GLYAT	glycine-N-acyltransferase	159.9
DOK5	docking protein 5	156.7
MMP11	matrix metallopeptidase 11 (stromelysin 3)	151.9
SMC4	structural maintenance of chromosomes 4	150.4
PCDH7	protocadherin 7	146.0
CST2	cystatin SA	141.3
GRIA3	glutamate receptor, ionotropic, AMPA 3	137.2
OMD	osteomodulin	135.7
FKBP2	FK506 binding protein 2, 13kDa	134.9
COL10A1	collagen, type X, alpha 1	134.8
KERA	keratocan	133.2
ANO3	anoctamin 3	131.6
SLC22A20	solute carrier family 22, member 20	130.9
C1QTNF3	C1q and tumor necrosis factor related protein 3	130.2
AFAP1	actin filament associated protein 1	126.3
CCDC80	coiled-coil domain containing 80	118.9
MPPED2	metallophosphoesterase domain containing 2	114.9
HBG1	hemoglobin, gamma A	112.997
C9orf50	chromosome 9 open reading frame 50	112.782
HPS4	Hermansky-Pudlak syndrome 4	112.58
WNT7B	wingless-type MMTV integration site family, member 7B	112.485
OGN	osteoglycin	109.759
C2orf27A/C2orf27B	chromosome 2 open reading frame 27A	108.106
PTCHD4	patched domain containing 4	107.808
FGF10	fibroblast growth factor 10	106.813
P2RY12	purinergic receptor P2Y, G-protein coupled, 12	105.905
GAS7	growth arrest-specific 7	102.181
ALLC	allantoicase	100.672
DIO2	deiodinase, iodothyronine, type II	98.68
PYGO1	pygopus homolog 1 (Drosophila)	98.043
CCL15	chemokine (C-C motif) ligand 15	97.136
PXDNL	peroxidasin homolog (Drosophila)-like	94.701
THSD7B	thrombospondin, type I, domain containing 7B	94.367
MYL1	myosin, light chain 1, alkali; skeletal, fast	92.013
HOXB8	homeobox B8	90.856
F9	coagulation factor IX	90.807
KRT6B	keratin 6B	90.019
AJAP1	adherens junctions associated protein 1	89.109
GJB2	gap junction protein, beta 2, 26kDa	86.414
TG	thyroglobulin	86.269
FNDC1	fibronectin type III domain containing 1	85.559

Genes are listed in order of the fold change in mRNA expression level between the patients with CHP and the contorl.

well as with poor survival, which is in accordance with our results (5). Since BAL and surgical lung biopsy are invasive and sometimes intolerable procedures, our results suggest that monitoring the changes in FVC or DLco may be sufficient to predict the outcome of patients with CHP. To the best of our knowledge, this is the first time that the predictive ability of serial changes in FVC or DLco in patients

Table 5. IPA analysis (Top 5 Functional and canonical pathway analyses)

Functional analysis / Name	P-value	Number of molecules
Diseases and Disorders		
Neurological Disease	6.29E-09 - 1.00E-03	60
Inflammatory Response	2.44E-06 - 3.92E-03	196
Infectious Disease	8.28E-06 - 5.05E-04	72
Cancer	1.10E-05 - 3.89E-03	597
Connective Tissue Disorders	3.21E-05 - 3.85E-03	109
Molecular and Cellular Functions		
Cell Death and Survival	8.34E-09 - 3.89E-03	344
Cell-To-Cell Signaling and Interaction	2.20E-08 - 3.39E-03	170
Cellular Function and Maintenance	2.27E-07 - 3.54E-03	180
Cellular Movement	4.38E-07 - 3.91E-03	217
Cellular Development	5.26E-07 - 3.92E-03	274
Physiological System Development and Function		
Tissue Development	2.20E-08 - 2.91E-03	139
Hematological System Development and Function	5.22E-07 - 3.92E-03	243
Tissue Morphology	5.22E-07 - 3.59E-03	189
Immune Cell Trafficking	5.31E-07 - 3.10E-03	140
Hematopoiesis	3.96E-06 - 3.54E-03	128

Canonical Pathway analysis / Name	p-value	Ratio
TNFR1 Signaling	6.65E-04	10/54 (0.185)
Integrin Signaling	1.22E-03	24/208 (0.115)
TNFR2 Signaling	1.58E-03	7/34 (0.206)
TREM1 Signaling	2.36E-03	10/75 (0.133)
Molecular Mechanisms of Cancer	2.91E-03	35/387 (0.09)

with CHP has been demonstrated. Based on these findings, we believe that it is important to further investigate the molecular background of patients with CHP who show a greater annual decline in FVC, in order to clarify the molecular pathogenesis of CHP and to identify potential therapeutic targets.

Among the several highly upregulated genes listed in Table 4, transmembrane/secretory genes such as *CST1*, *SFRP4*, *EFNA2*, *DOK5*, *MMP11*, *PCDH7*, *CST2*, and *WNT7B* may be useful biomarkers and potential therapeutic targets. Among them, MMP11 is one of the members of the matrix metalloproteinase family that may play an important role in the pathogenesis of pulmonary fibrosis through extracellular matrix remodeling, basement-membrane breakdown, epithelial cell apoptosis, cell migration, and angiogenesis (41). EFNA2 is a cell surface glycosyphosphatidylinositol (GPI)-bound ligand for ephrin receptors, a family of receptor tyrosine kinases, which are crucial for migration and adhesion during neuronal, vascular, and epithelial development (42). To verify the biological and clinicopathological significance of the candidate gene products, further validation of their expression at protein level in the lung tissues and loss-of-function assays, using siRNA, are warranted. Further, evaluation of their usefulness as a potential diagnostic serum biomarker by enzyme-linked immunoassay (ELISA) systems is also necessary (17).

Interestingly, IPA demonstrated that several pathways related to inflammatory responses and immunological diseases were differentially expressed in patients with rapidly progressive CHP (Table 5). Among the most prominent pathways in rapidly progressive CHP, there are several interesting pathways related to inflammatory and autoimmune diseases, such as TNFR signaling and TREM1 signaling pathways, which is consistent with the results of several previous investigations (43). Further, several agents that inhibit the TNFR signaling pathway, such as adalimumab, infliximab, etanercept, golimumab, and certolizumab, are available (44). Although a recent clinical trial using soluble TNF-alpha receptor agonist has failed to improve survival in patients with IPF (45), such agents that alter inflammatory pathways may be beneficial for patients with rapidly progressive CHP but not for patients with IPF. Further clinical studies are required to determine whether these agents can actually alter the progression of CHP.

In this study, the nine patients with rapidly progressive CHP were divided into two groups according to their transcriptional profiles (Figure 2), although no significant difference was identified in the clinical backgrounds between these groups (Table 3). These results may suggest the possibility of inter-individual heterogeneity in the molecular pathogenesis of rapidly progressive CHP. To further investigate this possibility, patients with stable CHP without progression should also be included in the gene expression analysis. However, in clinical practice, such stable patients tend to be followed up without surgical lung biopsy; therefore, we could not include these patients in the present gene expression analysis.

Although this study showed promising results, it has some limitations. First, this study was conducted in a retrospective manner. Therefore, some information such as changes in DLco was not obtained from all the patients studied during follow-ups. Second, the number of patients included in the study was not sufficient for a valid statistical analysis. Further prospective studies are required to clarify whether the annual decline in lung function can predict the prognosis of patients with CHP in a large multi-institutional setting. Third, only Japanese patients were studied. Considering the ethnic differences in the occurrence of drug-induced interstitial pneumonia and acute exacerbation in patients with IPF (46, 47), the application of these results to non-Japanese patients should be carefully extrapolated.

In conclusion, the greater annual decline in FVC and/or DLco is an independent predictor of the poorer prognosis of patients with CHP. Further, genes and pathways related to inflammatory responses and autoimmune diseases have been demonstrated to be differentially expressed in patients with rapidly progressive CHP as compared to the controls. The findings of this study could offer a powerful strategy for rapid identification and further evaluation of target molecules for personalized treatment of patients with rapidly progressive CHP.

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