Role of Deleterious Rare Alleles for Acute-Onset Diffuse Interstitial Lung Disease in Collagen Diseases

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ABSTRACT

OBJECTIVE: Acute-onset diffuse interstitial lung disease (AoDILD) includes acute exacerbation of interstitial lung disease (ILD), drug-induced ILD, and *Pneumocystis* pneumonia in collagen diseases patients. As AoDILD causes a poor prognosis in collagen disease patients, the pathogenesis of AoDILD should be investigated. Exome sequencing studies revealed that rare variants were detected to be causative in some diseases. Recently reported upregulated genes in acute exacerbation of idiopathic pulmonary fibrosis could provide candidate genes for restricted exome analysis of AoDILD in collagen disease. Here, we investigated rare variants in the coding and boundary regions of these candidate genes in AoDILD.

METHODS: Deleterious rare variants in the coding and boundary regions of the candidate genes were analyzed by exome sequencing and the deleterious rare allele frequencies in AoDILD were compared with those of controls.

RESULTS: A significant association was detected for deleterious rare alleles in *NPL* ($P = .0044$, $P_c = .0399$, odds ratio [OR] = 10.05, 95% confidence interval [CI] = 3.01-33.55). A deleterious rare allele frequency in the 9 candidate genes ($P = .0011$, OR = 7.17, 95% CI = 2.80-18.33) was also increased in AoDILD in multigene panel analysis. The Krebs von den Lungen–6 (KL-6) levels in AoDILD patients with deleterious rare alleles were tended to be lower than those without ($P = .0168$, $P_c = .1509$).

CONCLUSIONS: The deleterious rare alleles in *NPL* were associated with AoDILD. In addition, the deleterious rare allele frequency in the 9 candidate genes was also increased in AoDILD. The deleterious rare alleles might contribute to the pathogenesis of AoDILD.

KEYWORDS: Collagen disease, AoDILD, rare allele

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Introduction

Collagen diseases including rheumatoid arthritis (RA), systemic sclerosis (SSc), or polymyositis/dermatomyositis (PM/DM) are frequently associated with interstitial lung disease (ILD). Interstitial lung disease influences the prognosis of collagen diseases.[1,2] Acute-onset diffuse ILD (AoDILD) occurs in patients with collagen diseases.[3-5] Acute exacerbation of ILD, drug-induced ILD, and *Pneumocystis* pneumonia were included in AoDILD, but they are sometimes overlapping and it is often difficult to distinguish these 3 conditions. Acute-onset diffuse interstitial lung disease is empirically treated with corticosteroid. Although the precise mechanisms of AoDILD

are still unknown, the pathogenesis of AoDILD might be explained by the immune reconstitution inflammatory syndrome caused by the latent or apparent infection of *Pneumocystis jirovecii* or other undetected organisms.[6] The immune reconstitution inflammatory syndrome was reported to be occurred in collagen disease patients treated with immunosuppressive reagents.[7,8] As the prognosis of AoDILD is quite poor, it is necessary to clarify the precise pathogenesis of AoDILD.

Recent exome sequencing studies revealed that deleterious rare variants including loss-of-function variants (nonsense variants, frameshift variants, or splice site variants) and deleterious missense variants (variants changing amino acid residues on positions conserved in orthologs) were detected to be causative in some diseases.[9-14] Gene expression profiles in lung from patients with acute exacerbation of idiopathic pulmonary fibrosis were analyzed[15] and some genes were reported to be upregulated. These upregulated genes could be candidates for restricted exome analysis to reveal the genetic predisposition for the induction of AoDILD. Here, we investigated rare variants in the coding and boundary regions of these candidate genes in AoDILD patients and tried to compare the frequencies of deleterious rare alleles of these patients with those of controls.

Materials and Methods

Patients and controls

A total of 30 patients with collagen diseases were admitted to Sagamihara Hospital because of AoDILD requiring corticosteroid pulse therapy. Acute-onset diffuse interstitial lung disease was defined as acute or subacute onset and progression within a month, the presence of hypoxia, clinical symptoms (fever, dry cough, or dyspnea), and findings for ILD in chest computed tomography.[3,4] Patients with evidence of apparent heart disease or bacterial infection were excluded. The criteria include the acute exacerbation of ILD and acute interstitial pneumonia without already-existing ILD. These 30 collagen disease patients with AoDILD were native Japanese living in Japan and satisfied the American College of Rheumatology criteria for SSc,[16] RA,[17] or Bohan criteria for PM/DM.[18] Diagnoses of the patients included 27 RA, 1 SSc, and 2 PM/DM. These 30 patients with AoDILD survived during the periods of hospitalization and include 3 acute exacerbation of ILD (1 SSc and 2 PM/DM), 27 drug-induced ILD (27 RA, treated with methotrexate: 27, bucillamine: 5, sulfasalazine: 1, gold sodium thiomalate: 1, tacrolimus: 1, infliximab: 2, or etanercept: 5), and 0 *Pneumocystis* pneumonia. The definition of *Pneumocystis* pneumonia, drug-induced ILD, and acute exacerbation of ILD was previously described.[4] The allele frequencies of candidate genes in Japanese population were referred to 3.5KJPN panel from Tohoku Medical Megabank Organization genome cohort study (https://ijgvd.megabank.tohoku.ac.jp/).[19] The range of the age and sex of these Japanese controls was reported elsewhere (https://ijgvd.megabank.tohoku.ac.jp/statistics/statistics-3-5kjpn-all/). This study was reviewed and approved by Sagamihara National Hospital Research Ethics Committee (2008121012) and University of Tsukuba Research Ethics Committee (156-5). Written informed consent was obtained from all study participants. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Exome sequencing followed by restricted candidate gene analyses

Genomic DNA was captured by SureSelectXT Human All Exon Kit V6 (Agilent Technologies, Santa Clara, CA), followed by sequencing on Hiseq 2500 (Illumina, San Diego, CA). Sequence reads were mapped to *Homo sapiens* genome assembly of GRCh37/hg19 by GeneData Expressionist for Genomic Profiling V9.1.4a (Genedata, Basel, Switzerland). Variants found in the coding and boundary regions of the candidate genes were analyzed. Variants with minor allele frequencies equal to or more than 1% in 3.5KJPN panel were excluded.[14] Synonymous variants and intronic variants outside of splice site regions were also excluded. Missense variants, nonsense variants, frameshift variants, and splice site variants were included in the remaining variants. Deleterious missense variants (probably damaging or possibly damaging in PolyPhen-2 HumDiv and disease causing in Mutation Taster) were defined by the protein prediction algorithms of PolyPhen-2 HumDiv (http://genetics.bwh.harvard.edu/pph2/index.shtml)[20] and Mutation Taster (http://www.mutationtaster.org/).[21] Allele numbers of deleterious rare variants, that is, deleterious missense variants, nonsense variants, frameshift variants, and splice site variants, in the candidate genes were counted for the following statistical analyses.

Statistical analysis

The deleterious rare allele frequency in each gene or multigene panel in AoDILD patients was compared with that in Japanese controls by Fisher exact test using 2×2 contingency tables under the allele model.[9,10] The clinical manifestations of AoDILD patients with deleterious rare alleles were compared with those without by Mann–Whitney *U* Test or Fisher exact test using 2×2 contingency tables. The corrected P (P_c) value was calculated for correction of multiple testing by Bonferroni method.

Results

Associations of deleterious rare alleles in candidate genes with AoDILD

Exome sequencing was conducted with an average sequencing depth of 71.9 and at least 10-fold sequencing read coverage was achieved for 98.9% of the target sequences. Candidate

Table 1. Burden of deleterious rare alleles in the AoDILD patients and controls.

	CASE (2N = 60)	CONTROL (2N = 7104)	*P*	OR	P_C	95% CI
NPPA	1 (1.7)	7 (0.1)	0.0651	17.18	0.5859	2.08-141.87
NPL	3 (5.0)	37 (0.5)	0.0044	10.05	0.0399	3.01-33.55
PGAP1	1 (1.7)	17 (0.2)	0.1406	7.07	NS	0.93-53.96
SEC24A	0 (0.0)	17 (0.2)	1.0000	3.35	NS	0.20-56.30
DEFA4	0 (0.0)	0 (0.0)	NA	NA	NA	NA
DEFA3	0 (0.0)	0 (0.0)	NA	NA	NA	NA
SLC25A37	0 (0.0)	7 (0.1)	1.0000	7.82	NS	0.44-138.48
TMOD2	0 (0.0)	4 (0.1)	1.0000	13.04	NS	0.69-244.88
HILPDA	0 (0.0)	0 (0.0)	NA	NA	NA	NA
Multigene panel analysis	5 (8.3)	89 (1.3)	0.0011	7.17		2.80-18.33

Abbreviations: AoDILD, acute-onset diffuse interstitial lung disease; CI, confidence interval; NA, not applicable; NS, not significant; OR, odds ratio.
Allele frequencies are shown in parenthesis (%). Deleterious rare allele frequencies of AoDILD patients were compared with those of Japanese controls by Fisher exact test using 2 × 2 contingency tables under the allele model. The corrected *P* (P_c) value was calculated for correction of multiple testing by Bonferroni method.

genes were selected from the results of the gene expression profiles of acute exacerbations of idiopathic pulmonary fibrosis.[15] From 20 upregulated gene probes in acute exacerbations, essential genes including histon-related genes, heat shock protein genes, and deoxyribonuclease genes were excluded and 9 candidate genes remained (Table 1). When variants with minor allele frequencies equal to or more than 1% in 3.5KJPN, synonymous variants, and intronic variants outside of splice site regions were excluded, 5 missense variants and 1 splice site variant were found in the 9 genes of 30 AoDILD patients. Of these 5 missense variants, 4 variants were predicted to be deleterious (probably damaging or possibly damaging in PolyPhen-2 HumDiv and disease causing in Mutation Taster). These 4 deleterious missense variants include 1 in *NPPA* (c.280G>A, chr1:11907340, p.Gly94Arg), and 3 in *NPL* (c.133A>G, chr1:182772897, p.Asn45Asp, rs193141545; c.319G>T, chr1:182783948, p.Gly107Cys; c.364G>C, chr1:182783993, p.Asp122His). One splice acceptor deletion variant in *PGAP1* (c.1221-2delA, chr2:197750200) also remained. In total, 5 alleles of these 5 deleterious rare variants were detected in 30 AoDILD patients (2 alleles in the patients with acute exacerbation of ILD and 3 in drug-induced ILD).

Deleterious rare allele frequencies in the 9 candidate genes of the AoDILD patients and the Japanese controls are presented in Table 1. The deleterious rare allele frequency in *NPL* ($P = .0044$, $P_c = .0399$, odds ratio [OR] = 10.05, 95% confidence interval [CI] = 3.01-33.55) was increased in AoDILD. The deleterious rare allele frequency in the 9 candidate genes ($P = .0011$, OR = 7.17, 95% CI = 2.80-18.33) was also increased in AoDILD in multigene panel analysis.

Demographic features of the AoDILD patients with or without deleterious rare alleles in the 9 candidate genes

The clinical features of AoDILD patients with or without deleterious rare alleles in the 9 candidate genes were compared (Table 2). The Krebs von den Lungen–6 (KL-6) levels in AoDILD patients with deleterious rare alleles in the 9 genes were tended to be lower than those without ($P = .0168$, $P_c = 0.1509$).

Discussion

This study showed that deleterious rare alleles in *NPL* were associated with AoDILD. *NPL* gene encodes N-acetylneuraminate pyruvate lyase that regulates cellular concentrations of N-acetyl-neuraminic acid. It was reported that compound heterozygous mutations in *NPL* gene caused sialuria, skeletal myopathy, and cardiac myopathy.[22] Catabolic products of N-acetylneuraminate pyruvate lyase are N-acetyl glucosamine and N-acetyl mannosamine. As the potential relationship between variants in *NPL* gene and AoDILD is still unknown, it remains obscure whether these catabolic products could rescue AoDILD. The KL-6 levels in AoDILD patients with deleterious rare alleles in the 9 genes were tended to be lower than those without (Table 2). As KL-6 is a sialylated carbohydrate antigen, detected levels of KL-6 could be decreased in individuals with deleterious rare alleles of *NPL*. We found increased frequencies of deleterious rare alleles in the upregulated candidate genes in AoDILD patients, suggesting the causal effects of these genes.

To the best of our knowledge, this is the first study reporting the predisposition of deleterious rare alleles in *NPL* for AoDILD. In addition, the deleterious rare allele frequency in

Table 2. Comparison of the demographics between AoDILD patients with or without deleterious rare alleles.

	DELETERIOUS RARE ALLELE (+)	DELETERIOUS RARE ALLELE (–)	*P*	P_C
Number	5	25		
Male, No. (%)	2 (40.0)	8 (32.0)	1.0000[a]	NS
Mean age, y (SD)	56.8 (10.2)	68.7 (7.8)	0.0391	0.3517
Diagnosis of RA, No. (%)	3 (60.0)	24 (96.0)	0.0640[a]	0.5764
Mean KL-6, U/mL (SD)	311.7 (100.8)	1032.8 (765.9)	0.0168	0.1509
Mean SP-D, ng/mL (SD)	158.8 (211.6)	191.1 (170.1)	0.2801	NS
β-D-glucan, pg/mL (SD)	11.7 (7.6)	6.6 (1.5)	0.0780	0.7021
Rheumatoid factor positive, No. (%)	5 (100.0)	24 (96.0)	1.0000[a]	NS
Age at onset of underlying collagen diseases, y (SD)	40.3 (12.1)	55.3 (11.1)	0.0696	0.6265
Current or past smokers, No. (%)	0 (0.0)	11 (50.0)	0.2300[a]	NS

Abbreviations: AoDILD, Acute-onset diffuse interstitial lung disease; KL-6, Krebs von den Lungen-6; RA, rheumatoid arthritis; SP-D, surfactant protein-D.
Average values or numbers were shown. Standard deviations or percentages were shown in parenthesis. Association was analyzed between AoDILD patients with or without deleterious rare alleles by Mann–Whitney *U* Test or Fisher exact test using 2 × 2 contingency tables.
[a]Fisher exact test was used. The corrected *P* (P_c) value was calculated for correction of multiple testing by Bonferroni method.

the 9 candidate genes was also increased in AoDILD. There are several limitations in this preliminary study. Because of the low frequencies of deleterious alleles, the small number of candidate genes, and the limited sample size in this study, 5 deleterious rare alleles were found in AoDILD and the modest association was detected. The AoDILD patients in this study were heterogeneous and include 3 acute exacerbation of ILD and 27 drug-induced ILD. This is a single center study performed in Japanese populations. The predisposition should be confirmed in future large scale multicenter and multiethnic studies based on the data of whole exome analyses.

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Author Contributions

HF and ST conceived and designed the experiments. HF and SO performed the experiments. HF analyzed the data. HF, KS, AH, AK, TM, NF, and ST contributed reagents/materials/analysis tools. HF and ST contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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