Viral Infection in Acute Exacerbation of Idiopathic Pulmonary Fibrosis

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At a Glance Commentary

Scientific Knowledge on the Subject: The etiology of acute exacerbation of idiopathic pulmonary fibrosis remains unknown. Occult viral infection has been proposed as one possible cause of acute exacerbation of idiopathic pulmonary fibrosis.

What This Study Adds to the Field: This study uses the most current genomics-based technologies to investigate the possible infectious etiology of acute exacerbations of idiopathic pulmonary fibrosis. The majority of cases demonstrate no evidence of viral infection. Torque teno virus was present in a significant minority of cases, as well as cases of acute lung injury. The clinical significance of this finding remains to be determined.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org
ABSTRACT

Rationale: Idiopathic pulmonary fibrosis is a progressive, uniformly fatal interstitial lung disease. An acute exacerbation of idiopathic pulmonary fibrosis is an episode of acute respiratory worsening without an identifiable etiology. Occult viral infection has been proposed as a possible cause of acute exacerbation.

Objectives: To use unbiased genomics-based discovery methods to define the role of viruses in acute exacerbation of idiopathic pulmonary fibrosis.

Methods: Bronchoalveolar lavage and serum from patients with acute exacerbation of idiopathic pulmonary fibrosis, stable disease, and acute lung injury were tested for viral nucleic acid using multiplex polymerase chain reaction, pan-viral microarray, and high-throughput cDNA sequencing.

Results: Four of forty-three acute exacerbation of idiopathic pulmonary fibrosis patients had evidence of common respiratory viral infection (Parainfluenza (n=1), Rhinovirus (n=2), Coronavirus (n=1)); no viruses were detected in the bronchoalveolar lavage from stable patients. Pan-viral microarrays revealed additional evidence of viral infection (herpes simplex virus (n=1), Epstein-Barr virus (n=2), and torque teno virus (TTV) (n=12)) in acute exacerbation patients. TTV infection was significantly more common in acute exacerbation patients than stable controls (p=0.0003), but present in a similar percentage of acute lung injury controls. Deep sequencing of a subset of acute exacerbation cases confirmed the presence of TTV but did not identify additional viruses.
Conclusions: Viral infection was not detected in the majority of acute exacerbation of idiopathic pulmonary fibrosis cases. TTV was present in a significant minority of cases, as well as cases of acute lung injury; the clinical significance of this finding remains to be determined.

Abstract Word Count: 249

Keywords: acute lung injury; virus; infection; pulmonary fibrosis; etiology
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease with no known cause or cure (1,2). While some patients experience a steady progression of disease over time, many have periods of relative stability punctuated by episodes of acute respiratory worsening that can be fatal (3,4). When no identifiable cause for this acute worsening is found, it is termed an acute exacerbation of IPF (5).

Acute exacerbation of IPF is defined as an idiopathic acute worsening of dyspnea characterized radiologically by the presence of bilateral ground glass abnormality on high-resolution computed tomography (HRCT) scan of the chest (5). It is estimated that between 5 and 10% of patients with IPF will experience an acute exacerbation annually, with more physiologically advanced disease at higher risk (4,5,6). It remains unclear whether acute exacerbation of IPF represents a primary acceleration of the underlying fibroproliferative process in IPF or is a clinically occult secondary complication (e.g. infection) (5,7).

Acute exacerbation of IPF is often accompanied by fever, increased cough, and myalgia, suggesting an infectious etiology. Respiratory viruses have been considered a particularly likely cause, based on the similarities in clinical and radiological presentation between acute exacerbation of IPF and viral pneumonitis and the poor sensitivity of standard methods of viral detection (5). Also, there may be an increased incidence of acute exacerbation of IPF in East Asia, a finding that could be explained by an environmental factor such as an endemic virus.
Preliminary evaluations of the role of infection in acute exacerbation have yielded mixed results (11,12).

In this study, we tested the hypothesis that acute exacerbation of IPF is caused by occult viral infection. We prospectively collected bronchoalveolar lavage (BAL) from patients experiencing acute exacerbation of IPF and controls (stable IPF and acute lung injury (ALI)) and used multiplex polymerase chain reaction (PCR), and a pan-viral microarray discovery platform to test for the presence of known and novel viruses. Next-generation parallel sequencing (deep sequencing) was applied to a subset of acute exacerbation samples to increase the sensitivity of viral detection.

**METHODS**

**Study population**

Patients with acute exacerbation of IPF were identified prospectively from two centers (University of Ulsan, Korea; Tosei General Hospital, Japan). Sequential subjects with acute exacerbation of IPF were enrolled if they underwent bronchoscopy and were consented. Diagnostic criteria for acute exacerbation of IPF were prespecified according to established criteria (5). All acute exacerbation patients had negative clinical evaluation for infectious causes including routine bacterial and viral BAL antibody titers and cultures for respiratory syncytial virus (RSV), influenza A and B, human parainfluenza viruses (HPIVs), adenovirus, human cytomegalovirus, herpes simplex, and varicella-zoster virus. Control patients with stable IPF (defined by the absence of acute exacerbation) and ALI were identified from an existing cohort.
at a single center (University of Ulsan, Korea) and underwent bronchoscopy at the time of diagnosis. IPF and ALI were defined by consensus criteria (1,13,14). BAL from a case of IPF that was positive for Rhinovirus and CMV was included as a blinded positive control. All centers received approval from their institutional review board or equivalent, and all patients provided informed consent.

**Sample collection and processing**

In all cases, bronchoscopy was performed as part of patients’ clinical evaluations. In most cases, BAL was collected within the first 48 hours of admission to the hospital. In general, BAL was performed in a single sub-segment of the right middle lobe or lingula, with at least 100 milliliters of sterile saline instilled. Blood was not collected as part of the initial study protocol. A subset of acute exacerbation samples and stable IPF controls underwent phlebotomy at the time of bronchoscopy as part of a separate ongoing repository study. These samples were available to us for analysis. All samples were stored at -80 degrees Celsius until ready for processing. Total RNA was extracted from 200 microliters of each sample using the RNeasy mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions.

**PCR analysis**

A blinded, nested respiratory multiplex PCR was run on the BAL samples from acute exacerbation of IPF and stable IPF controls for pre-specified respiratory viruses (influenza virus, HPIV, RSV, human rhinovirus, human enterovirus, human coronavirus, human metapneumovirus, and human adenovirus) (15). PCR was also carried out to confirm viral signatures present on pan-viral microarray analysis (herpes simplex virus (HSV), Epstein-Barr
virus (EBV), and torque teno virus (TTV)) using published nested primer sets (16,17,18). Additional PCR for TTV was performed on BAL samples from ALI controls and on serum samples from a subset of acute exacerbation of IPF and stable IPF controls in an effort to better define the epidemiology of TTV.

**Pan-viral microarray analysis**

Acute exacerbation of IPF samples and stable IPF controls were randomly amplified to generate cDNA which was hybridized blindly to a pan-viral microarray as previously described (19). Arrays were scanned using the Axon 4000B scanner and intensities were calculated using GenePix 6.0 (Molecular Devices, Sunnyvale, California). The presence of a viral signature was determined using Cluster 3.0 (20) and E-predict (21).

**Deep sequencing analysis**

BALs from a subgroup of study patients with acute exacerbation of IPF were selected for deep sequencing on the Illumina Genome Analyzer IIx platform (Illumina, San Diego, CA) based on the presence of both fever and myalgia, symptoms suggestive of a viral-like illness. Deep sequencing libraries were prepared and analyzed as described in the online data supplement.

**Statistical methods**

Clinical data are expressed as means or percentages, unless otherwise stated. The primary comparison was between BAL samples from acute exacerbation of IPF and stable IPF controls. Additional comparisons were made between BAL samples from acute exacerbation of IPF and ALI controls, and serum samples from acute exacerbation of IPF and stable IPF controls. In all
cases, intergroup comparisons were performed conservatively using non-parametric methods (Wilcoxon signed-rank test) and Chi-squared/Fishers exact analyses as appropriate. Regression analysis was performed to determine the relationship between clinical factors, PCR positivity, and survival. Clinical data analysis was performed using SAS 9.1 (SAS Institute, Cary, NC). Statistical significance was defined as a p-value < 0.05.

RESULTS

Patient characteristics
Sixty patients with acute exacerbation of IPF (52 Korean, 8 Japanese) were identified between 2006 and 2009. Forty-three of these underwent bronchoscopy and were enrolled in the study. The median time from diagnosis to acute exacerbation was 85 days. Their clinical characteristics are summarized in Table 1. Twelve (28%) of the acute exacerbation of IPF patients presented with both fever and myalgia, suggestive of a viral-like illness. Forty patients with stable IPF, and twenty-nine patients with ALI were included as controls. Patients with ALI predominantly had lower respiratory tract infection (90%) as the underlying cause for their lung injury. These patients were all mechanically ventilated and had a mean PaO2/FiO2 ratio of 228. Serum was available from 22 acute exacerbation of IPF patients and 31 stable IPF controls.

Viral detection by PCR and pan-viral microarray
Four acute exacerbation of IPF BAL samples (9%) were positive for common respiratory viruses by initial multiplex PCR (two for rhinovirus, one for human coronavirus-OC43, and one for parainfluenza virus-1). All stable IPF samples were negative for common respiratory viruses (p
= 0.12 compared to acute exacerbation of IPF samples, Table 2). Array analysis of acute exacerbation of IPF BAL samples revealed the presence of torque teno virus (TTV) and several human herpesviruses, as well as accurately identifying rhinovirus and CMV in the known positive control. To pursue these findings further, we carried out sensitive genome-specific PCR reactions for HSV, EBV and TTV. This yielded 15 additional BAL positives in acute exacerbation of IPF samples (Table 3). Of these additional viruses, only TTV was significantly more common in acute exacerbation of IPF compared to stable controls (28% vs 0%, p=0.0003). Four BAL samples revealed double infections: two with TTV and rhinovirus, one with TTV and parainfluenza virus-1, one with TTV and HSV. One BAL sample revealed a triple infection of TTV, EBV, and coronavirus. Overall, 14 (33%) of acute exacerbation of IPF samples were positive for virus compared to no positives in the stable IPF samples (p < 0.0001). There was no difference in the frequency of fever and myalgia between virus positive and virus negative cases, and there was no significant difference in the use of corticosteroid treatment.

Viral detection by deep sequencing

BALs from twelve of the study patients with acute exacerbation of IPF were selected for deep sequencing to investigate the possibility of viruses being present but undetected by PCR and microarray. Of these samples, two were PCR-positive for tested viruses—one for TTV and one for TTV and HSV. After initial quality filtering, approximately 26 million pairs, or 52 million total reads, comprised the primary dataset. Each of the 12 barcoded acute exacerbation of IPF samples was represented with at least 3 million high quality reads. Over 98% of the reads were derived from human origin, and of the remaining reads, approximately 0.1% were recognizably bacterial in origin. Only a few hundred were potentially attributable to known non-human
eukaryotes and viruses. Aside from bacteriophages, only three viruses (two TTVs and one HSV) were found, consistent with the PCR results. After all stages of mapping to a sequence database were finished, approximately 0.6% of the original dataset remained without attribution.

**Comparison of TTV positive and TTV negative acute exacerbation of IPF samples**

There were no significant differences in age, gender, baseline pulmonary function, or time to bronchoscopy in TTV positive acute exacerbation of IPF patients compared to TTV negative acute exacerbation of IPF patients (data not shown). TTV positive patients appeared sicker, with 58% requiring mechanical ventilation (vs. 29% in TTV negative patients, p = 0.09) and 75% dying at 60 days (vs. 42% in TTV negative patients, p = 0.06). Overall median survival time in the TTV positive patients was 29 days (vs. 88 days in TTV negative patients, p = 0.19, Figure 1). Bivariate regression analysis of potential independent predictors of survival time (presence or absence of prednisone treatment at the time of BAL, mechanical ventilation and the time of BAL, and TTV positivity on BAL) revealed only mechanical ventilation as statistically significant (hazard ratio (HR) 2.30, p = 0.03). TTV positivity was not an independent predictor of survival time (HR 1.65, p = 0.20).

**TTV positivity in acute exacerbation of IPF and stable IPF serum**

Six of 22 patients (27%) with acute exacerbation of IPF were PCR positive for TTV in serum, compared to five of 31 patients (16%) with stable IPF (p = 0.34). There was no relationship between serum and BAL positivity. Three of the six acute exacerbation patients (50%) that were PCR positive in serum were also PCR positive in BAL. Four of the 16 acute exacerbation patients (25%) that were PCR negative in serum were PCR positive in BAL.
TTV positivity in ALI BAL

The statistically significant link between BAL-associated TTV and acute exacerbation of IPF prompted us to also examine BAL samples from 29 patients with ALI. TTV was detected in seven of 29 BAL samples (24%) from ALI patients; this was not significantly different from the prevalence of TTV in BAL samples from acute exacerbation of IPF (28%, p = 0.73).

DISCUSSION

Using highly-sensitive PCR, pan-viral microarrays, and deep sequencing technologies in a large, well-described cohort of patients with acute exacerbation of IPF and controls, we found that the majority of cases of acute exacerbation of IPF had no evidence of an underlying viral infection. This suggests that viral infection is not a common cause of acute exacerbation of IPF.

Overall, we found viral nucleic acid in the BAL of 33% of patients with acute exacerbation of IPF; no viruses were found in samples from stable IPF controls. There were two rhinovirus positive samples, one coronavirus positive sample, and one parainfluenza virus positive sample, suggesting that a small minority (9%) of acute exacerbations of IPF may be caused by occult infection with common respiratory viruses. Surprisingly, the most common virus detected in the BAL of acute exacerbation of IPF patients was TTV, which was present in 28% of acute exacerbation BAL samples. This finding was not unique to acute exacerbation of IPF, as 24% of BAL samples from ALI controls were also TTV positive.

Two recent studies have commented indirectly on the possible role of occult viral infection in acute exacerbation of IPF. The first study performed gene expression microarrays on whole lung
tissue from eight patients who died of acute exacerbation of IPF, 23 patients with stable IPF, and 15 healthy controls (12). The authors concluded that acute exacerbation of IPF was characterized by a pattern of enhanced epithelial injury and proliferation, but found no gene expression profiles indicative of a response to viral or bacterial infection. In a second study of 27 patients presenting with acute decline in fibrotic lung disease (13 of whom had confirmed acute exacerbation of IPF), five had antigenic or PCR evidence of viral infection (one parainfluenza virus, two HSV, and two CMV infections), three of which were missed on standard viral culture (11).

Our study expands significantly on previously published reports. First, we take an unbiased approach to viral discovery using cutting-edge genomic methodology. It is the first study to do this in acute exacerbation of IPF. Our use of sequencing to confirm all suspected viruses rules out the possibility of spurious PCR results, a common pitfall of the technique. Second, our large cohort of well-defined acute exacerbation patients with adequate controls allows for greater certainty regarding our conclusions. Third, we have identified an unexpected virus (TTV) that was associated with 33% of acute exacerbations, and that was absent in stable IPF.

The pathogenetic significance of TTV in acute exacerbation of IPF BAL is unclear. TTV is a non-enveloped single stranded circular DNA virus that exists in a genetically diverse clade (22,23). The virus seems to have broad tissue tropism as it has been detected in peripheral blood mononuclear cells (PBMCs) as well as bone marrow, spleen, liver, and lung (22). Infection with TTV in the human population is worldwide, with prevalences of viremia ranging from 8%-80% depending upon the population studied and detection methodology employed. When only considering the hemi-nested PCR of the N22 region used in this study, rates of TTV DNA found
in healthy blood donors range from 8.4% to 12% (24, 25) and do not appear to correlate with the geographic location of the patients. Most infected subjects are asymptomatic, and to date efforts to link TTV viremia with any acute or chronic pathologic state have been unsuccessful (22). Although there have been reports of TTV in the upper respiratory tract (nasopharynx and oral cavity) (26), TTV has not been identified in BAL fluids. TTV has previously been detected in the serum of 12 of 33 Japanese patients (36%) with IPF. In this study, TTV appeared more frequently in cases that progressed to acute exacerbations, and TTV positivity was suggested to correlate with worse survival (27). Our findings do not show a correlation between the presence of TTV in the serum and the presence of TTV in the BAL, nor any correlation between serum TTV positivity and a diagnosis of acute exacerbation.

It is possible that de novo TTV infection in the lung causes direct alveolar epithelial cell injury and acute respiratory worsening. If so, this process does not appear to be unique to acute exacerbation of IPF, since we detected TTV at a similar frequency in BAL from patients with ALI. While this does not exclude a potential role for TTV in the pathogenesis of acute exacerbation of IPF, it is also compatible with the idea that inflammation or injury in the lung may non-specifically trigger local TTV replication, or may result in increased vascular permeability in the lung allowing circulating virus to enter the alveolar compartment. In the latter two cases, the presence of TTV would represent a consequence of lung inflammation rather than its cause. The idea that local TTV replication might be enhanced by underlying inflammatory signaling is supported by in vitro studies of PBMCs from TTV negative donors (28). These PBMCs were infected in vitro with TTV, cultured with and without the presence of phytohemagglutinin, lipopolysaccharide, and interleukin-2, and then examined for evidence of TTV replication. In this experiment, TTV mRNA and replicative intermediates were only found
in the stimulated PBMCs, consistent with an infection-amplifying role for inflammatory signaling.

The methodologies used in this study have unparalleled sensitivity for viral detection. The multiplex nested PCR is several fold more sensitive than virus culture and direct immunofluorescent tests, with the ability to amplify less than 10 copies of target nucleic acid (15). For viral discovery, however, PCR is of limited utility as it identifies only *a priori* viral targets. Pan-viral microarray precludes the need for a preconceived list of targets, although even with its proven sensitivity, its benefit is dependent on the signal to noise ratio of the nucleic acid (19). The use of deep sequencing to look further for evidence of viral infection in a high-risk sub-population of acute exacerbation patients therefore adds confidence to our results, as it produces an unbiased, high-resolution description of the microbial landscape of the sample tested. This technology has been used to identify novel viruses in human diarrhea, and to describe the microbiome of the distal gut (31,32), but never in BAL (29,30). In the current study, two samples subjected to deep sequencing were positive for known viruses by PCR and pan-viral array screening. Using an efficient and sensitive pipeline for sorting reads, these positive PCR findings were confirmed, and no additional viruses were detected in these or the other samples tested. Interpretation of these findings must be tempered by the fact that existing computational methods for recognizing potential viral genomes are imperfect, and may fail to identify novel agents with only limited homology to known viral genera. The same is true of array-based viral detection methods.

One important limitation of this study is the potential for false-negative results due to the timing of sample collection. BAL was performed early in the course of hospitalization, most commonly
in the first 48 hours after admission, and the median time from symptom onset to sampling was 7 days. Importantly, no difference in the time from symptom onset to sample collection was found between virus positive and virus negative cases. The duration of replicating virus in BAL is largely unknown, and it is possible that a virus could have stopped shedding during this time. In this study, we have maximized our likelihood of detecting virus by obtaining BAL samples early after admission and using highly-sensitive viral detection techniques.

In summary, this study used unbiased, highly sensitive genomics-based discovery methods to investigate the role of viral infection in a large, well-characterized cohort of patients with acute exacerbation of IPF. The results of this study suggest that the majority of cases of acute exacerbation of IPF are not due to viral infection. Future research into the etiology of acute exacerbation of IPF should confirm these findings, further investigate the role of TTV, and consider other possible occult complications (e.g. aspiration) that may cause acute respiratory worsening in these patients.
ACKNOWLEDGEMENTS

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Figure Legends

**Figure 1:** Survival time in TTV positive acute exacerbation of IPF patients compared to TTV negative acute exacerbation of IPF patients. Mean survival time was 29 days vs 88 days, respectively, $p = 0.19$. 
Table 1: Clinical Characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Acute</th>
<th>Stable</th>
<th>ALI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exacerbation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 43</td>
<td>n = 40</td>
<td>n = 29</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>65</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td>Male gender %</td>
<td>88</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td>Surgical lung biopsy %</td>
<td>21</td>
<td>28</td>
<td>NA</td>
</tr>
<tr>
<td>Smoking %</td>
<td>84</td>
<td>75</td>
<td>48</td>
</tr>
<tr>
<td>Baseline FVC %</td>
<td>73</td>
<td>79</td>
<td>NA</td>
</tr>
<tr>
<td>Baseline DLCO %</td>
<td>60</td>
<td>70</td>
<td>NA</td>
</tr>
<tr>
<td>Mechanical ventilation, %</td>
<td>37</td>
<td>NA</td>
<td>100</td>
</tr>
<tr>
<td>Immunosuppressive therapy, %</td>
<td>60</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Immunosuppressive therapy includes corticosteroids with or without immunomodulator therapy.

Abbreviations: FVC % = forced vital capacity percent predicted; DLCO % = diffusing capacity for carbon monoxide percent predicted.
Table 2: Respiratory viral detection in acute exacerbation and stable IPF

<table>
<thead>
<tr>
<th>Virus</th>
<th>Acute</th>
<th>Stable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exacerbation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 43</td>
<td>n = 40</td>
<td></td>
</tr>
<tr>
<td>Any respiratory virus</td>
<td>4 (9%)</td>
<td>0 (0%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>2 (5%)</td>
<td>0 (0%)</td>
<td>0.49</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Parainfluenza</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>--</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>--</td>
</tr>
<tr>
<td>Influenza</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>--</td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>--</td>
</tr>
<tr>
<td>RSV</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>--</td>
</tr>
</tbody>
</table>

Abbreviations: RSV = respiratory syncytial virus.
### Table 3: Array-based viral detection in acute exacerbation and stable IPF

<table>
<thead>
<tr>
<th>Virus</th>
<th>Acute Exacerbation</th>
<th>Stable</th>
<th>Acute lung injury</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 43</td>
<td>n = 40</td>
<td>n = 29</td>
<td></td>
</tr>
<tr>
<td>TTV</td>
<td>12 (28%)</td>
<td>0 (0%)</td>
<td>7 (24%)</td>
<td>0.0003</td>
</tr>
<tr>
<td>EBV</td>
<td>2 (5%)</td>
<td>0 (0%)</td>
<td>NA</td>
<td>0.49</td>
</tr>
<tr>
<td>HSV</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>NA</td>
<td>1.0</td>
</tr>
<tr>
<td>CMV</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NA</td>
<td>--</td>
</tr>
</tbody>
</table>

* p value is for comparison of acute exacerbation to stable control.

Abbreviations: TTV = torque teno virus; EBV = Epstein-Barr virus; HSV = herpes simplex virus; CMV = cytomegalovirus.
ONLINE DATA SUPPLEMENT for:

Viral Infection in Acute Exacerbation of Idiopathic Pulmonary Fibrosis

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Deep sequencing library preparation

Libraries were prepared as previously described (E1). In brief, nucleic acid extracted from BAL was first primed using abbreviated Illumina A and B adaptors attached to a unique 3-bp sequence tag (barcode) followed by a random hexamer. The 3-bp barcode was incorporated into each of the twelve samples to allow for 12-plex barcoded sequencing within a single lane on the Illumina flow cell. cDNA was amplified for 25 cycles of PCR using the barcoded adaptors, and the PCR product was run on a 4% native polyacrylamide gel at 4C to select for a narrow size distribution centered around 250-bp. The amplicons were then precipitated with 100% ethanol at 4C and resuspended in 16 microliters of water. Two microliters were carried into a second round of PCR amplification using the abbreviated A adaptor and a full length B adaptor for 15 cycles using 22-bp of the 3’ end of the Illumina A adaptor and 61-bp of the Illumina B adaptor as primers. The product was size selected once more for products around 304-bp, which would carry the correct A/B topology. Ethanol precipitated DNA was then PCR amplified for ten cycles using the full length Illumina A adaptor and the 5’ end of the B adaptor. This final library was sequenced on one lane of a paired end deep sequencing run with 65-bp read from each end of the insert.

Deep sequencing analysis

Low complexity reads with inadequate Lempel-Ziv-Welch (LZW) compression ratios were removed, and barcodes indicated by the sequence of the first 3-bp of each read were used to bin reads according to their original sample (E2). These reads were first filtered for host sequence through a high stringency BLAT to the human genome and transcriptome (E3), then screened for the presence of non-human sequence through iterative BLAST analysis to the NCBI NT database
(E4). First, high identity hits were isolated using a MEGABLAST with a word size of 28 against NT. Reads that did not align significantly to NT with a high word size were then aligned to NT again using MEGABLAST with word size of 12, followed by a sensitive BLASTN alignment with a word size of 7 and an e-value of 1e-3. All hits at every step were sorted according to NCBI taxonomy identifiers.
ONLINE DATA SUPPLEMENT REFERENCES


