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Metagenomic Sequencing Detects Respiratory Pathogens in Hematopoietic Cellular Transplant Patients

To the Editor:

Lower respiratory tract infections (LRTIs) are a leading reason for hospitalization and mortality in hematopoietic cellular transplant (HCT) recipients (1). Despite this, the etiologic pathogens often remain unidentified because of reduced yields of traditional diagnostics in the setting of prophylactic antimicrobials, reduced antibody titers, and infections from uncommon opportunistic microorganisms. Metagenomic next-generation sequencing (mNGS) may offer enhanced diagnostic capabilities by providing culture-independent, comprehensive measurement of the microbial composition of clinical samples (2, 3). By capturing both microbial and human RNA, mNGS also permits simultaneous transcriptional profiling of the host immunologic response and can deliver rapid (<48 h) and actionable microbiologic data for precise infectious disease diagnosis (2).

As a result of the clear need for enhanced LRTI diagnostics in HCT recipients, we sequentially enrolled 22 adult HCT recipients hospitalized for acute respiratory illnesses who underwent bronchoscopy and BAL between January 25, 2012, and May 20, 2013, under University of Michigan protocol HUM00043287. Standard-of-care BAL microbiologic testing was uniformly performed on all patients and included semiquantitative cultures for bacteria, mycobacteria, fungi, and cytomegalovirus; *Aspergillus* galactomannan assay; silver stain for *Pneumocystis jirovecii*; multiplex polymerase chain reaction influenza A/B, respiratory syncytial virus and human metapneumovirus; and human herpesvirus-6 polymerase chain reaction, as detailed in the Methods in the online supplement (4). Additional diagnostics were performed on blood and nasopharyngeal samples at the discretion of the treating physicians, as described in Table 1 and detailed in the online supplement (4).

RNA and DNA sequencing libraries were constructed from 250 μl BAL from each patient and underwent paired-end Illumina sequencing according to established methods (4). Pathogen detection leveraged a custom bioinformatics pipeline that discriminates pathogens from background microbial contaminants in clinical samples (2, 3). A ranking score consisting of the nucleotide reads aligned per million reads sequenced (rpM) multiplied by the sum of the nt and nr *Z*-scores for each genus relative to no-template controls was used [score = $\mathrm{rpM}_{nt} \times (Z_{nt} + Z_{nr})$] (4).

Microbes identified were classified as confirmed pathogens if *1*) both clinical testing and mNGS identified the microbe, *2*) there existed literature evidence of pathogenicity in the lungs, and *3*) the score was as least twofold greater than that of any other microbe of the same type (virus, bacteria, or fungus) identified in the patient. Microbes were considered new potential pathogens if mNGS alone identified the microbe and criteria 2 and 3 described here were met; all other microbes were considered unlikely or uncertain pathogens. Findings were independently confirmed by viral PCR or bacterial 16S rRNA gene sequencing, as described (4).

Standard-of-care clinical diagnostics identified microbes in seven patients (32%), six of which were considered pathogens by treating physicians, and all of which were detected by mNGS (Table 1). In addition, mNGS identified well-recognized respiratory pathogens in six patients with otherwise negative testing, including human coronavirus 229E and human rhinovirus A (HRV-A); the former, despite being a leading cause of community-acquired pneumonia (5), is still not included as a target on many clinical respiratory viral PCR panels. With respect to potential bacterial pathogens, mNGS identified *Streptococcus mitis* in patient 13, an oropharyngeal microbe known to cause bacteremia and acute respiratory distress in HCT recipients (6), and

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Table 1. Clinical and Microbiologic Data

ID	Symptoms	Duration	Chest Radiography	Standard BAL Microbiology	Top-Scoring Microbes by mNGS	Non-BAL Microbiology	Prophylactic Antimicrobials	Treatment Antimicrobials	GVHD	Respiratory Support	Cause of Death
Pathogens identified by conventional and NGS diagnostics											
1	Fever, hypoxia	3 d	RLL, LLL infiltrate	HMPV	HMPV	HMPV (NP), *C. difficile* (stool)	ACV, LEVO, MICA, TMP-SMZ	CFPM, VANC	aGVHD, cGVHD	NC	Relapse, Day 253
5	Cough, dyspnea, fever	3 d	Multifocal nodules, bronchiectasis	*Stenotrophomonas maltophilia*	*S. maltophilia* + HRV-A	Negative	ACV, LEVO, TMP-SMZ, VORI	TMP-SMZ, VANC	aGVHD, cGVHD	NC	GVHD, Day 462
8	Dyspnea	12 d	B/L GGO	RSV	RSV	CMV (blood)	ACV, LEVO, PENT, POSA	CIDV, FOSC, GCV, VANC, (IVIG)	No	HFNC	Relapse, Day 265
9	Cough	3 d	RUL, RML, LUL GGO, nodules	Negative	HMPV	HMPV (NP)	ACV, TMP-SMZ	None	cGVHD	None	Alive
10	Cough, fever	4 d	Bibasilar airspace disease	RSV	RSV	RSV (NP)	FLUC, LEVO, vACV	CFPM, RIBV, (IVIG)	aGVHD, cGVHD	NC	GVHD, Day 3,024
36	Dyspnea, fever	1 d	Diffuse septal thickening, GGO	HHV-6	HHV-6	HHV-6 (blood)	ACV, FLUC, LEVO, VORI	FOSC	No	None	Alive
New potential pathogens identified by NGS											
6	Cough, dyspnea	3 d	B/L nodules	Negative	HCOV-229E	Negative	TMP-SMZ, vACV, VORI	CFPM, TOBR, VANC	cGVHD	None	Alive
7	Dyspnea	1 d	B/L perihilar opacities	Negative	HCOV-229E	Negative	ACV, FLUC, LEVO, TMP-SMZ	CFPM, VANC, (IVIG)	aGVHD, cGVHD	IPPV × 4 d	GVHD, Day 96
13	Cough, dyspnea	2 d	LLL opacities, air trapping	Negative	*Corynebacterium propiniquum*	Negative	ACV, FLUC, LEVO, TMP-SMZ	CFPM, VANC	No	IPPV × 3 d	Idiopathic respiratory failure, Day 51
14	Cough, fever	2 d	LLL opacities	Negative	HRV-A	Negative	TMP-SMZ	None	cGVHD	None	Alive
18	Cough, fever	2 d	RML, RLL atelectasis	Negative	HRV-A	*S. epidermidis* (blood)	FLUC, LEVO, vACV	CFPM, VANC	n/a	None	Alive
19	Dyspnea	4 wk	RUL, LUL reticular opacities	Negative	*Streptococcus mitis*	Negative	ACV, LEVO, VORI	VANC	aGVHD, cGVHD	None	Alive

(Continued)

Table 1. (*Continued*)

ID	Symptoms	Duration	Chest Radiography	Standard BAL Microbiology	Top-Scoring Microbes by mNGS	Non-BAL Microbiology	Prophylactic Antimicrobials	Treatment Antimicrobials	GVHD	Respiratory Support	Cause of Death
Microbes of uncertain or unlikely pathogenicity											
3	Fever	2 d	B/L infiltrates	Negative	*Flavobacterium sp.*	Gram-positive rod (blood)	ACV, LEVO, TMP-SMZ, VORI	VANC	No	None	GVHD, Day 267
11	Cough, fever	2 d	LLL opacities	Negative	Negative	Negative	ACV, FLUC, LEVO, TMP-SMZ	CFPM	aGVHD, cGVHD	None	Alive
22	Dyspnea, rigors	1 d	RUL, LUL peribronchial thickening, GGO	Negative	Torque teno virus	Negative	ACV, LEVO, TMP-SMZ, VORI	CFPM	cGVHD	NC	Alive
23	Cough, dyspnea, fever	3 wk	B/L GGO, lymphadenopathy	Negative	Negative	Epstein-Barr virus viremia	ACV, LEVO, MICA, TMP-SMZ, VORI	CFPM	aGVHD	IPPV × 10 d (until death)	No autopsy, Day 10
24	Dyspnea, fever	1 wk	B/L perivascular and airspace GGO	Negative	*Pyrenophora sp.*	Negative	ACV, TMP-SMZ, VORI	CFPM, TOBR	aGVHD, cGVHD	NC	Alive
25	Cough, dyspnea, fever	4 wk	Diffuse bronchiectasis, multifocal GGO with nodules	Negative	WU polyoma virus	Negative	LEVO, VORI, TMP-SMZ	VANC	aGVHD, cGVHD	NC	Alive
31	Dyspnea	2 wk	B/L GGO, bronchiectasis	Negative	*Prevotella sp.*	Negative	ACV, FLUC, LEVO, TMP-SMZ	CFPM	cGVHD	NC	Alive
34	Cough, dyspnea	2 wk	Multifocal nodules, opacities, air trapping	Negative	Torque teno virus	Negative	ACV, TMP-SMZ	None	cGVHD	None	Alive
35	Cough, dyspnea	2 d	Multifocal GGO, tree-in-bud opacities	Negative	Negative	Negative	ACV, FLUC, LEVO	VANC	aGVHD, cGVHD	NC	Alive
37	Cough, dyspnea	8 d	B/L GGO	CMV*	CMV*	Negative	TMP-SMZ	None	aGVHD, cGVHD	None	Alive

Definition of abbreviations: ACV = acyclovir; B/L = bilateral; CFPM = cefepime; CIDV = cidofovir; CMV = cytomegalovirus; FOSC = foscarnet, FLUC = fluconazole; GCV = ganciclovir; GGO = ground-glass opacities; GVHD = graft-versus-host disease (a, acute; c, chronic); HCOV = human coronavirus; HFNC = high-flow nasal cannula; HHV-6 = human herpesvirus-6; HMPV = human metapneumovirus; HRV-A = human rhinovirus A; IPPV = invasive positive pressure ventilation; IVIG = intravenous immunoglobulin; LEVO = levofloxacin; LLL = left lower lobe; LUL = left upper lobe; MICA = micafungin; mNGS = metagenomic next-generation sequencing; n/a = not applicable; NC = nasal cannula; NGS = next-generation sequencing; NP = nasopharyngeal; PENT = pentamidine; POSA = posaconazole; RIBV = ribavirin; RLL = right lower lobe; RML = right middle lobe; RSV = respiratory syncytial virus; RUL = right upper lobe; TMP-SMZ = trimethoprim-sulfamethoxazole; TOBR = tobramycin; vACV = valacyclovir; VANC = vancomycin; VORI = voriconazole.
Chest computed tomography was obtained for all subjects except 1, 3, 4, 8, 9, and 14, who received chest X-rays.
*Clinicians concluded that CMV in patient 37 was not the principal cause of respiratory disease.

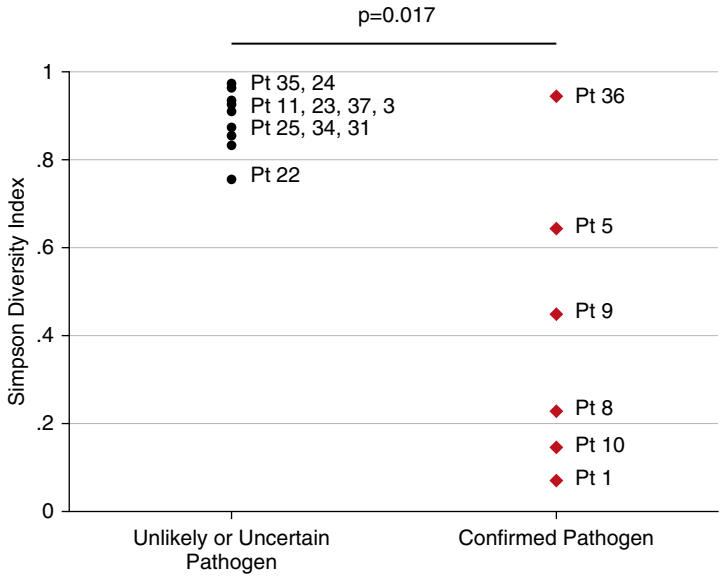

Figure 1. Bronchoalveolar lavage microbial diversity is inversely associated with presence of a transcriptionally active respiratory pathogen. Each data point represents a single patient (Pt) for whom the Simpson diversity index is plotted on the *y*-axis. Subjects are grouped according to confirmed pathogen (red diamonds) vs. unlikely or uncertain pathogen (black circles). Patients with confirmed pathogens had significantly lower diversity relative to patients with only microbes of unlikely pathogenicity (median, 0.34 [interquartile range, 0.15–0.64; $n = 6$] vs. 0.92 [interquartile range, 0.86–0.93; $n = 10$]; $P = 0.017$).

Corynebacterium proprinquum, one of the few virulent *Corynebacterium* species associated with LRTI (7). mNGS identified a diversity of DNA viruses including human herpesvirus-6, cytomegalovirus, herpes simplex virus, Epstein-Barr virus, human papilloma virus, and torque teno viruses; however, only five of these also had well-defined evidence of active replication marked by detectable RNA transcripts (Table 1). mNGS identified microbes of uncertain pathogenicity in nine patients (Table 1) who had coexisting clinical diagnoses of graft-versus-host disease (patients 11, 22, 23, 24, 25, 31, 34, 35, and 37) or bacteremia/sepsis (patient 3), which could have contributed to respiratory symptoms resulting from noninfectious pulmonary inflammation.

Because asymptomatic carriage of respiratory pathogens is well described (8), establishing biomarkers of genuine infection is critical for determining the significance of a given microbiologic finding. Loss of diversity within respiratory tract microbial communities has been proposed as an ecological marker of infection (9), and we thus evaluated alpha diversity of microbes identified by RNA sequencing and found that subjects with confirmed pathogens had a significantly lower Simpson's diversity index than patients with microbes of unlikely or uncertain pathogenicity (median, 0.34 [interquartile range (IQR), 0.15–0.64] vs. 0.92 [IQR, 0.86–0.93], Wilcoxon rank sum $P = 0.017$; Figure 1) (4).

We next hypothesized that the host airway immunologic response might serve as a biomarker of infection. To test this idea, we evaluated *a priori*–selected gene sets related to innate and adaptive immune responses from the Molecular Signatures Database and found significantly increased expression in patients with confirmed LRTI pathogens versus those without (median, 94.9 [IQR, 93.8–105.6] vs. 33.1 [IQR, 20.7–75.1], Wilcoxon rank sum $P = 0.022$) (4, 10). We observed that two of the three HRV-A–positive subjects demonstrated the lowest expression of this immune response metric, whereas the remaining subject, who was coinfected with HRV-A and *Stenotrophomonas maltophilia*, had one of the highest values, consistent with prior reports demonstrating that HRV can induce a broad range of disease severity and that viral–bacterial coinfection can increase the severity of disease (8).

Although these results are highly encouraging, this proof-of-concept study suggests many routes for improvement. First, this study was limited by a relatively small sample size, only one case of culture-positive bacterial LRTI, no fungal infections, and no subjects with exclusively clinical diagnoses of noninfectious airway diseases. Second, our limited sequencing depth did not yield the human transcriptome coverage that would be desired for optimal differential gene expression analyses, although we were able to rigorously evaluate a composite metric of immunity genes. Future studies with larger cohorts are ongoing to validate the sensitivity and specificity of mNGS for LRTI diagnosis in this population and develop robust gene classifiers that can distinguish LRTI from noninfectious airway diseases in HCT recipients.

In summary, we leverage continued improvements in metagenomic sequencing to expand the capabilities of LRTI diagnostics in HCT recipients with acute respiratory illnesses. We demonstrate that compared with current microbial diagnostics, mNGS has a greater capacity for detecting microbes and an ability to couple pathogen detection with simultaneous profiling of the host response and the airway microbiome. ■

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Peripheral and Alveolar Cell Transcriptional Programs Are Distinct in Acute Respiratory Distress Syndrome

To the Editor:

Our understanding of transcriptomic responses in acute respiratory distress syndrome (ARDS) is derived, almost exclusively, from studies that have used samples procured from the peripheral circulation as opposed to the alveolar space (1–4). However, it is not known to what degree genomic signatures captured from circulating leukocytes accurately reflect the gene expression patterns of leukocytes in the lung. We hypothesized that the transcriptional signals from peripheral blood monocytes (PBMs) and alveolar macrophages (AMs) would be distinct in ARDS, and that alterations in the transcriptional state of immune cells in the lung would provide new insights into the pathogenesis of ARDS. Some of the results of these studies have been previously reported in the form of an abstract (5).

Methods

Subjects enrolled in the Phase II Randomized Placebo-controlled Trial of Omega-3 Fatty Acids for the Treatment of Acute Lung Injury trial (6) conducted between 2006 and 2008 were included in this study. We performed genome-wide expression analysis of total RNA isolated from paired AM and PBM samples purified from BAL fluid and peripheral blood specimens, respectively, collected from patients within 48 hours of the diagnosis of ARDS. Negative selection for AMs and PBMs was achieved by incubating cells with antibody-labeled microbeads containing the following markers: CD3, CD15, CD19, CD235a, CD294, and CD326. We did not use antibodies for CD294 (eosinophils) and CD326 (epithelial cells) for the blood samples because mononuclear cells were isolated from whole blood before antibody incubation via polyester gel centrifugation. RNA extracted from isolated cells was assessed for purity, and then hybridized to an Illumina HumanRef-8 BeadChip that was inclusive of 18,415 unique genes.

We performed variance stabilization and quantile normalization of the raw microarray data, using the Bioconductor package *lumi* (7). Detailed microarray information has been deposited at Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89953). Principal component analysis was performed on the basis of whole-genome gene expression variability between paired AM and PBM samples (8). Differential gene expression between the two cell populations was determined by a Bayesian implementation of the *t* test (http://cybert.microarray.ics.uci.edu) on $\log_2$-transformed probe intensities, using a sliding window size of 101 and a Bayesian confidence estimate value of 10.

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