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Research Paper

Nasopharyngeal Protein Biomarkers of Acute Respiratory Virus Infection

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ABSTRACT

Infection of respiratory mucosa with viral pathogens triggers complex immunologic events in the affected host. We sought to characterize this response through proteomic analysis of nasopharyngeal lavage in human subjects experimentally challenged with influenza A/H3N2 or human rhinovirus, and to develop targeted assays measuring peptides involved in this host response allowing classification of acute respiratory virus infection. Unbiased proteomic discovery analysis identified 3285 peptides corresponding to 438 unique proteins, and revealed that infection with H3N2 induces significant alterations in protein expression. These include proteins involved in acute inflammatory response, innate immune response, and the complement cascade. These data provide insights into the nature of the biological response to viral infection of the upper respiratory tract, and the proteins that are dysregulated by viral infection form the basis of signature that accurately classifies the infected state. Verification of this signature using targeted mass spectrometry in independent cohorts of subjects challenged with influenza or rhinovirus demonstrates that it performs with high accuracy (0.8623 AUROC, 75% TPR, 97.46% TNR). With further development as a clinical diagnostic, this signature may have utility in rapid screening for emerging infections, avoidance of inappropriate antibacterial therapy, and more rapid implementation of appropriate therapeutic and public health strategies.

1. Introduction

Acute respiratory viral (ARV) infections are among the most common reasons for patient visits in primary and acute care settings (Hong et al., 2004; Johnstone et al., 2008). Many viruses cause such acute respiratory illness including human rhinovirus (HRV), respiratory syncytial virus (RSV) and influenza. These viruses can be associated with a range of clinical severity from asymptomatic to mild, self-limited illness to respiratory failure and death. Influenza alone causes 25 to 50 million infections annually in the USA, resulting in several hundred thousand hospitalizations and 20–40,000 deaths (Thompson et al., 2010).

Despite viral etiologies driving most cases of acute respiratory infection, definitive diagnostic tools for these syndromes are lacking. Even highly sensitive pathogen-specific tests such as PCR are dependent upon proper sampling technique and inclusion of virus-type-specific reagents and processing methods. Moreover, detection of a specific microbe in a clinical sample does not necessarily indicate the cause of the acute clinical syndrome. For example, it has been reported that HRV has been detected in up to 44% of asymptomatic individuals (Byington et al., 2015; Johnston et al., 1993). Therefore, better tools that help providers define the etiology

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of a suspected infectious syndrome in a safe, rapid, accurate, and cost-effective manner are of paramount importance for both individual and public health as recently noted by the Presidential Advisory Council on Combating Antibiotic-Resistant Bacteria (House, 2014), and others (O'Neill, 2015; Organization, 2015). A complementary diagnostic strategy to pathogen detection could focus on utilizing the varied (but pathogen-class specific) host-response to infection (Ramilo and Mejias, 2009; Zaas et al., 2014). This approach discriminates between infection and colonization. It is pathogen-agnostic and therefore circumvents another limitation of pathogen detection assays, which due to technical limitations are only capable of detecting a limited subset of microorganisms. Furthermore, categorizing infection based on host response provides additional insights into the mechanisms of infection and disease response, and may offer new targets, pathways, or strategies for therapeutic intervention.

We recently identified gene expression patterns in peripheral whole blood capable of differentiating (Zaas et al., 2009; Woods et al., 2013; McClain et al., 2016; Tsalik et al., 2016; Huang et al., 2011) individuals with symptomatic infection due to influenza H3N2, HRV, or RSV from uninfected individuals with >90% accuracy. Moreover, this ARV signature was validated in an independent population of patients with influenza A infection, demonstrating an ability to distinguish from bacterial respiratory infections (93% accuracy) and healthy controls (100% accuracy) (Zaas et al., 2009). Thus, host derived biomarkers are capable of making these types of distinction. However, considering the technical challenges inherent in developing peripheral blood host gene expression classifiers as a diagnostic tool – including semi-invasive venipuncture, RNA instability, processing complexity, relatively high cost of RNA profiling, and time to result – we sought to extend this host response paradigm for ARV diagnosis to an alternative and potentially more suitable sample matrix and analyte class.

Upon contact with the respiratory epithelium, respiratory viruses incite activation of type I interferons (IFNs) and pro-inflammatory cytokines, orchestrate proliferation of inflammatory cells and the innate immune response, and regulate induction of adaptive immunity (Yoneyama and Fujita, 2010; Koyama et al., 2008; Bhoj et al., 2008). Based on the prominent role of the nasopharyngeal epithelium in mediating ARV infections, we hypothesized that nasopharyngeal lavage (NPL) would reflect the in situ host response and serve as a potential target for diagnostic development. Furthermore, the NPL protein fraction represents an accessible sample matrix, providing a highly tractable diagnostic analyte class. Multiple reaction monitoring (MRM), a quantitative mass spectrometry (MS) platform for facile development of multiplexed, quantitative assays for measuring specific protein levels in biologic fluids and is routinely used for biomarker verification in clinical cohorts (Kiyonami et al., 2011; Gerszten et al., 2010; Boja and Rodriguez, 2011). In addition to being customizable for nearly any target protein, MRM assays provide a more specific quantitation of individual proteins and protein isoforms by targeting multiple unique peptides per protein target. Combined with internal stable-isotope labeled (SIL) peptide standards, these assays match or exceed the quantitative precision of ELISA assays with low femtomole limits of quantitation and analytical precision coefficient of variation < 10% across clinically sized cohorts (Addona et al., 2009; Aebersold et al., 2013).

Using human viral challenge cohorts for influenza A/H3N2 and HRV, we have discovered and independently verified multiple NPL protein biomarkers capable of classifying human influenza A and HRV infection from uninfected individuals. This work reinforces the important concept that host response to infection, particularly in the NPL proteome, serves as a potential basis for diagnostic testing. It also sheds light on the complex interactions of host and pathogen in two of the most common infectious diseases in humans.

2. Materials and Methods

2.1. Study Design

All pathogen exposures were approved by the relevant Institutional Review Boards and conducted according to the Declaration of Helsinki. All volunteers provided informed consent. The objective of these experimental challenge studies was to generate clinico-molecular classifiers of ARV infection through the development and characterization of high-density sample and data sets across the course of respiratory virus exposure, infection, and resolution. A description of methods used in each challenge study can be found in Supplementary materials and have been described previously (Liu et al., 2016; McClain et al., 2016; Woods et al., 2013; Zaas et al., 2009). Briefly, healthy volunteers underwent extensive pre-enrollment health screening and were excluded for positive baseline antibody titers of the strain of virus used in each challenge (Influenza A H3N2 A/Wisconsin/67/2005 or HRV serotype 39). Following 24–48 h in quarantine, we instilled viral inoculum into bilateral nares of subjects using standard methods. At predetermined intervals, biological samples and clinical and symptom data were collected. NPL sampling was performed daily for each participant. The H3N2 #2 cohort included an early (36 h post-inoculation) oseltamivir treatment arm, while HRV #2 included a blinded "sham" inoculation (saline only) control group. NPL analyses included baseline and time T samples from all individuals in each challenge study with complete and unambiguous symptomatology and microbiology data, and available NPL samples. Sample phenotype labels were blinded for MRM analysis, but were assayed in a manner to ensure that samples from an individual, and from within a challenge study, would be processed in same batch and assayed in close temporal proximity, to minimize batch effects between distinct phenotypes.

2.2. Case Definitions

Self-reported symptoms were recorded at predetermined intervals prior to inoculation and at least twice daily throughout the time-course of infection and resolution as reported previously (Zaas et al., 2009; Jackson et al., 1958) and described in Supplementary materials. This modified Jackson score requires subjects to rank 8 symptoms of upper respiratory infection (headache, sore throat, rhinorrhea, rhinitis, sneezing, coughing, myalgia, malaise) on a standardized scale of 0 (no symptoms) to 3 (high symptoms). Symptom scores were tabulated for each study participant to assign symptom status as symptomatic or asymptomatic (Supplementary Table S2A). For each symptomatic subject, time T was identified as time of maximal symptoms. The average time T was then defined for that cohort, which served as the time chosen for asymptomatic subjects (Table 1). Participants were tested for virus shedding based on quantitative culture assays as described previously

Table 1
Description of experimental ARV challenge cohorts. Four experimental HRV challenge cohorts (two influenza A/H3N2 and two HRV) are described, including adjudicated phenotype summary data for each. Individuals with discordant[a] symptom and shedding labels, i.e. symptomatic non-shedders, or asymptomatic shedders, are shown. Sx = symptomatic; Asx = asymptomatic; mean Sx and Asx time T represents the average time of maximal self-reported symptoms among subject included in NPL analysis.

Cohort	H3N2 #1	H3N2 #2	HRV #1	HRV #2
Subjects included in challenge	17	21	20	30
Subjects included in NPL analysis	15	21	20	24
Symptomatic	9	13	12	15
Shedding positive	10	10	12	15
Number discordant[a]	1	7	4	9
Mean Sx time T (in hrs)	69	68	66	76
Corresponding Asx time T (in hrs)	69.5	71	72	72

[a] Individuals with discordant symptom and shedding labels, i.e. symptomatic non-shedders, or asymptomatic shedders. NPL = Nasopharyngeal lavage. Sx = Symptomatic. Asx = Asymptomatic. Time T represents the time of maximal symptoms.

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