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Molecular classification of outcomes from dengue virus -3 infections

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ABSTRACT

Objectives: Dengue virus (DENV) infection is a significant risk to over a third of the human population that causes a wide spectrum of illness, ranging from sub-clinical disease to intermediate syndrome of vascular complications called dengue fever complicated (DFC) and severe, dengue hemorrhagic fever (DHF). Methods for discriminating outcomes will impact clinical trials and understanding disease pathophysiology.
Study design: We integrated a proteomics discovery pipeline with a heuristics approach to develop a molecular classifier to identify an intermediate phenotype of DENV-3 infectious outcome.
Results: 121 differentially expressed proteins were identified in plasma from DHF vs dengue fever (DF), and informative candidates were selected using nonparametric statistics. These were combined with markers that measure complement activation, acute phase response, cellular leak, granulocyte differentiation and viral load. From this, we applied quantitative proteomics to select a 15 member panel of proteins that accurately predicted DF, DHF, and DFC using a random forest classifier. The classifier primarily relied on acute phase (A2M), complement (CFD), platelet counts and cellular leak (TPM4) to produce an 86% accuracy of prediction with an area under the receiver operating curve of >0.9 for DHF and DFC vs DF.
Conclusions: Integrating discovery and heuristic approaches to sample distinct pathophysiological processes is a powerful approach in infectious disease. Early detection of intermediate outcomes of DENV-3 will speed clinical trials evaluating vaccines or drug interventions.

1. Background and objectives

DENV is an arthropod-borne RNA virus that causes significant health and/or economic impact to over 100 dengue-endemic countries in which approximately half of the world's population resides [1]. As a result, dengue fever (DF) is the most common vector-borne viral infection in humans [2]. Symptomatic DENV infections manifest as a flu-like disease characterized by a sudden onset of fever, maculopapular rash, and leucopenia [3]. A small group develop severe dengue disease (SDD), a later-onset syndrome characterized by vascular leakage and/or hemorrhage, leading to 500,000 annual hospitalizations with a case fatality rate of about 5% [4].

Risk factors for complicated DENV infections are multifactorial; these include host genetic factors [5–10], viral strains [11–13], anamnestic immune response from previous

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heterologous exposure [14–16], age [17,18] and/or gender effects [12]. Not surprisingly, then, the clinical manifestations of DENV infection is highly variable in terms of times of onset, involvement of thrombocytopenia [19,20], manifestations of endothelial injury/vascular leak [21], myositis, arthralgias and hepatic involvement [22,23]. As a result, the World Health Organization (WHO) refined dengue case classification schemes in 2009 to include the identification of non-severe dengue with early warning signs [24]. Accordingly, approaches seeking to discriminate acute, self-limited infections vs those that will progress to SDD have yielded inconsistent results [12,22,23,25].

Recent advances in global proteomic technologies enable more unbiased methods for disease discrimination than previously possible. Here we report, for the first time, a multi-level strategy for discovery and biomarker qualification of severity of DENV infections in a cohort of DENV-3 infections. This unique cohort represented a valid sampling of the entire infected population, including a balanced mixture of primary and secondary infections [26]. We tested the performance of robust, clinical grade quantitative proteomics measurements that sampled multiple pathophysiological processes, including complement activation, acute phase response, cellular leak, granulocyte differentiation and viral load, on clinical outcome. This study will be of value to produce objective case definition to better evaluate diagnostics and clinical interventions to reduce the impact of DENV-3 infections.

2. Study design

2.1. Study population and case definitions

Patients >5 years of age with suspected DF who were admitted to one of three hospitals in the city of Recife, Pernambuco, Brazil. After written informed consent (or assent) was obtained from each subject (or guardian) clinical and laboratory evaluations were performed on enrollment [33]. Dengue cases were classified according to the 1997 WHO Guidelines. DF was assigned by a high fever accompanied by at least two associated symptoms: headache, retro-orbital pain, myalgia, arthralgia and rash. DHF was defined by the same clinical manifestations as for DF, but with hemorrhagic manifestations, including a positive tourniquet test, thrombocytopenia (<100,000/mm^3), hemoconcentration or other sign of plasma leakage [24]. DFC was designated when subjects met the criteria for DF and who presented with hemorrhagic manifestations and thrombocytopenia (<100,000/mm^3), but the clinical/laboratory parameters did not fulfill the WHO DHF criteria as described by us earlier [26]. Infectious outcomes were 29.4% DF, 44% complicated DF (DFC), those with hemorrhagic or thrombocytopenia that did not meet the classical WHO criteria, and 8.2% DHF [26].

2.2. Biofluid analysis platform (BAP) fractionation and 2DE

The BAP pre-separation fractionation system is a semi-automated device to fractionate plasma into protein and peptide pools [12]. The protein pools were depleted of the most highly abundant proteins and labeled with BODIPY FL-maleimide (BD) [27,28]. BD-labeled proteins were separated by 2DE, imaged and analyzed using Progenesis/SameSpots software (Nonlinear Dynamics, Ltd., Newcastle Upon Tyne, UK).

2.3. Stable isotope dilution (SID)-selected reaction monitoring (SRM)-MS

Two technical replicates of analysis for each sample were analyzed by SID-SRM-MS. All of the SRM measurements had within-technical replicates coefficient of variation (CV) of 20% or less. To detect CFD, we developed an immuno-precipitation (IP)-SID-SRM-MS assay. An aliquot of 20 μL of plasma was immunoprecipitated with 4 μL of biotin conjugated anti-CFD Ab (Thermo Scientific, Waltham, MA). The samples were captured, washed and CFD abundance quantified with SID-SRM-MS.

Table 1
Clinical demographics.

Disease phenotype	Characteristic	Mean ± SE
DF (n = 59)	M/F	25/34
	Age (y)	22.7 ± 2.1
	Days of symptoms at sample 1	4.7 ± 0.26
	qPCR viral copies (per mL)	5.3E + 07 ± 2.0E + 07
	Platelet count (permL)	195,945 ± 6280.31*
	HCT (%)	39.3 ± 0.4*
DFC (n = 29)	M/F	17/12
	Age (y)	36 ± 3.16
	Days of symptoms at sample 1	5.1 ± 0.58
	qPCR viral copies (per mL)	4.5E + 10 ± 0.43E + 11
	Platelet count (per mL)	125,536 ± 6513.64*
	HCT (%)	41 ± 0.7*
DHF (n = 22)	M/F	7/15
	Age (y)	38.5 ± 4.43
	Days of symptoms at sample 1	5.4 ± 0.55
	qPCR viral copies (per mL)	1.70E + 08 ± 1.14E + 08
	Platelet count (per mL)	86,750 ± 5354.75
	HCT (%)	41.7 ± 1.0*

* $p < 0.05$ ANOVA.

2.4. Statistical analysis

Statistical comparisons were performed using SPSSv18 (SPSS, Inc., Chicago, IL). 2-way ANOVA, post-hoc testing using Tukey's t-test, and Kruskall–Wallis tests were applied as indicated. Student's t-tests were performed on the 2DE spots. The significance level in all instances was 0.05.

3. Results

The clinical parameters of the one hundred and ten (1 1 0) study volunteers are shown in Table 1. The three study groups were matched for age, gender [male/female (M/F)] distribution and days of fever. At the time of presentation, hematocrit (HCT) was significantly different between the DF and DHF diagnoses ($p = 0.046$, Tukey's t-test). The platelet concentration was also significantly different in all groups ($p < 0.001$, Tukey's t-test). These data suggested that the DFC was a distinct category of disease.

3.1. Discovery of candidate biomarkers related to DENV severity

We first applied discovery proteomics to identify any unique proteins using an unbiased protein profiling strategy (BAP; Methods). In this platform, plasma from a matched set of extremes (29 cases of DF and 22 cases of DHF) were subjected to analysis (Fig. 1). We detected 1311 protein spots after complete analysis; Student's t-tests were performed on the $\log_2$-transformed intensities for each. Of these 1311 spots, 121 were significantly different between the two classes ($p < 0.05$, Table 2). The top 10 canonical biological pathways dysregulated by DHF were identified by pathway enrichment analysis. The top 3 pathways included acute phase response signaling, LXR/RXR activation and coagulation system (Fig. 2). Pathways analysis of the dysregulated network showed an interconnected network enriched in acute phase response proteins, coagulation pathways, and cytoskeletal elements (Fig. 3).