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A Genomic Analysis of *Clostridium difficile* Infections in Blunt Trauma Patients

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Abstract

Background—Evidence demonstrates that susceptibility to *Clostridium difficile* infection (*C. diff.*) is related as much to host risk factors as bacterial potency. Using blood leukocyte genome-wide expression patterns of severe blunt trauma patients obtained by the NIGMS sponsored Glue Grant “*Inflammation and the Host Response to Injury*” we examined *C. diff.* patients’ leukocyte genomic profiles to determine pre- and post-infection gene expression changes.

Methods—The genomic responses of 21 severe trauma patients were analyzed (5 *C. diff.* 16 controls matched for age and severity of injury). After elimination of probe sets whose expression was below baseline or were unchanged, remaining probe sets underwent hierarchical clustering and principal component analysis. Molecular pathways were generated through Ingenuity Pathways Analysis®.

Results—Supervised analysis demonstrated 118 genes whose expression in *C. diff.* patients varied before and after their infection. Supervised analysis comparing *C. diff.* to matched non-*C. diff.* patients prior to infection suggested that the expression of 501 genes were different in the two groups with up to 87% class prediction ($p < 0.05$). Many of these genes are related to cell-mediated immune responses, signaling and interaction.

Conclusions—Genomic analysis of severe blunt trauma patients reveals a distinct leukocyte expression profile of *C. diff.* both prior to and after infection. We conclude that an association may exist between a severe trauma patient’s leukocyte genomic expression profile and subsequent susceptibility to *C. diff.* Further prospective expression analysis of this *C. diff.* population may

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reveal potential therapeutic interventions and allow early identification of *C. diff.* susceptible patients.

Level of Evidence—Level III prognostic/diagnostic study.

Background

In the hospitalized population, *Clostridium difficile* (*C.diff.*) infections are increasing in severity and incidence, as well as becoming more difficult to treat (1, 2). Over the past decade, *C.diff.* infections are more often associated with toxic megacolon, septic shock and death (3). In addition, medical care costs from this disease are historically high (1, 2). Although a significant percentage of hospitalized patients are colonized with the bacteria, most do not become infected (2). Well known risk factors for *C.diff.* associated diarrhea (CDAD) include advanced age, antibiotic exposure and hospital/nursing home exposure. Increasingly, evidence demonstrates that a patient's susceptibility to *C.diff.* infection is related as much to host risk factors as bacterial potency and environmental exposures. Evidence includes discovering a younger at risk population that has little or no antibiotic exposure and sometimes no previous hospital exposure (1, 3). In addition, certain individuals with a specific genetic polymorphism are at increased risk for primary and recurrent *C.diff.* infections (3–6).

The collaborative effort, “*Inflammation and the Host Response to Injury*” (Glue Grant), is a large-scale interdisciplinary research program funded by the National Institute of General Medical Sciences to better describe the different clinical outcomes after traumatic injury. The Trauma-Related DataBase (TRDB), a large multicenter database containing deidentified, prospectively collected clinical and gene expression data from patients with severe blunt trauma, was developed as a part of this program and has greatly facilitated research of clinical outcomes after trauma. A database of 2006 subjects with severe blunt trauma was compiled between 2003–2009, and, in a subset of 167 subjects, the Program collected serial genomic and proteomic data taken from whole blood leukocytes. The purpose of this study was to examine *C.diff.* trauma patient leukocyte genomic profiles to determine if there were common gene expression profiles in this population before and after infection.

Materials and Methods

Before initiation of this project, approval was obtained from the University of Florida Institutional Review Board to retrieve and analyze deidentified data obtained from the “*Inflammation and the Host Response to Injury*” TRDB.

Clinical Data/Epidemiological analysis

Data Source and Study Population—This is a multi-institution retrospective cohort study of clinical data derived from the “*Inflammation and the Host Response to Injury*” TRDB. Beginning November 2003, the “*Inflammation and the Host Response to Injury*” Program enrolled patients with severe blunt trauma from eight participating ACS designated level I trauma centers. A total of 2,006 blunt injured trauma patients who required transfusion and with a NISS>15 were analyzed from the Glue Grant database. Exclusion criteria included traumatic brain injury and an age less than 18 years old. Standard operating procedures (SOP) were followed by all participating institutions for clinical management to minimize variation for patient and data collection (7).

Genomics data analysis

All patients who were diagnosed with *C. diff.* and in whom genomic analysis was carried out with the HU-133plus v2 GeneChip™ were analyzed. Only one *C. diff.* patient who was studied using an enriched leukocyte subpopulation with a different microarray chip (HH/2 GeneChip™) was removed from analysis. The genomic responses of 21 patients were analyzed. This consisted of five patients diagnosed with CDAD. Subsequently, 16 matched controls by age, race/ethnicity, gender and Injury Severity Score (ISS) were identified for comparison. The mean array intensities of the Affymetrix HU133plus v2 Gene Chips™ were normalized among all the chips using dChip™. The entire normalized dataset of 55,381 probe sets was filtered to eliminate probe sets whose expression was below baseline in all samples. These remaining probe sets then underwent further statistical analysis including hierarchical clustering and principal component analysis using BRB Array Tools™. Leave one out cross validation was used to determine the probability of these probe sets being selected by chance using 1000 random permutations and Monte Carlo testing. For supervised genomic analysis of patients with CDAD before and after their infection, admission samples were compared to the first sample obtained after the patient's diagnosis of *C.diff* (n=5 for each group). For comparison of *C.diff.* to non-*C.diff.* patients after infection, the first sample collected after the diagnosis of *C.diff.* was used (n=5) and compared to admission samples of the non-*C.diff.* matched controls (n=16). Gene expression comparison of *C.diff.* patients to their matched controls prior to infection utilized the first sample obtained upon admission (n=5 and n=16, respectively). Signaling pathways were generated through Ingenuity Pathways Analysis®.

Results

Characteristics of patient microarray analysis

5 of 167 patients contained in the Glue Grant database who had microarray analyses were diagnosed with *C. diff.* during their hospitalization, again a prevalence relatively similar to the reported level in hospitalized patients (8). In addition, the percentage of patients who developed *C.diff.* (3%) is similar to that of 3.3% reported in the overall trauma population (3). 16 matched controls were identified from the TRDB based on age, gender, race/ethnicity and NISS score. Age and NISS were not statistically different. 2 out of 5 of the *C.diff.* and 5 of the 16 of the non-*C.diff.* patients were female and the average number of hospital days to diagnosis was 11 ± 5 .

Supervised analysis (threshold false discovery adjusted significance level of 0.001) demonstrated 118 genes that differentiated the same *C.diff.* patients (n=5) before and after their infection (Figure 1A), while 47 genes differentiated *C.diff* (n=5) after their infection to the non-*C.diff.* patients (n=16) at admission (Figure 1B). Interestingly, 501 genes were able to differentiate the two groups prior to infection, with up to 87% class prediction ($p < 0.01$) by leave one out cross validation analysis ($p < 0.05$) (Figure 1C). Ingenuity Pathways Analysis revealed that many of these genes are related to cell-mediated immune responses, signaling and interaction (Figure 2 and Table 1).

Discussion

Genetic variances that translate to immune differences have been reported to place the host at risk for CDAD (9, 10), making microarray analysis of *C.diff.* particularly relevant after trauma. The present analysis of the “*Inflammation and the Host Response to Injury*” database reveals several differences in the gene expression of peripheral leukocytes from severe trauma patients develop CDAD. As far as we are aware, this is the first report to demonstrate that the gene expression analysis of severe trauma patients at admission was

different in patients that subsequently develop *C.diff*. In addition, our data allows investigators to hypothesize that an individual's early genomic response to *C.diff*. might be associated with whether colonization evolves into infection and its subsequent severity.

Not surprisingly, our analysis reveals a specific gene expression pattern in the blood leukocytes of trauma patients after *C.diff*. as compared to before their infection, or as compared to patients without CDAD. However, few of these genes can be directly associated to proteins that explain the response to infection. Our work does demonstrate that a specific genomic profile may be present in this population prior to the patient's documented *C.diff*. infection, and many of the genes identified could play an important role in the vulnerability of the host to this pathogen (Table 1 and Figure 2). Further prospective analysis may allow early identification of at risk patients, as well as a better understanding of how/why *C.diff*. colonization transforms to infection, and hopefully lead to the creation of novel therapeutics.

Gene expression is being used as a predictive tool in the medical population, and leukocyte gene expression after trauma can predict patients who will develop multiple organ dysfunction (11). In fact, an individual's early gene expression profile can be used to determine their recovery, or lack thereof (11). Given the cost, morbidity and mortality of ICU patients, tools that isolate at risk populations and allow vigilant care are of particular use; thus, the preemptive determination of a patient's *C.diff* risk could benefit the populace. The incidence and severity of these infections has increased over the past decade, especially with the emergence of a new virulent strain (1). Also, the disease is evolving—younger patients with minimal antibiotic and hospital exposure have been identified (3). Rapid identification and interventions are crucial to a patient's recovery with *C diff*. 2). Attentive screening and preemptive treatment of those patients most at risk for *C.diff*. could improve outcomes as well as lower the costs of hospital LOS; CDAD is associated with, at minimum, an additional cost of \$3,600 (2). This concept is reinforced by the association of *C.diff*. with increased hospital/ICU LOS and ventilator days (2).

Although there are well identified external factors that contribute to infection by this bacterium, an individual's response to the presence of *C.diff*. might play a role with infection pathogenicity (1). Only some of the patients colonized with *C. diff*. develop an infection rather than remaining symptomless carriers (9). Data demonstrates this in part due to the host's IgG response to the bacterial toxins (9). Additionally, alterations in the interleukin (IL)-8 gene promoter, with subsequent cytokine over-production, is predictive for an increased risk for *C.diff*. infection, recurrent infection and an inappropriate antibody response to *C.diff*. toxin (4, 6, 10). IL-8 recruits and induces the transmigration of neutrophils into the intestinal lumen (10), which may create a pattern of excessive inflammation without appropriate adaptive immune responses. The admission leukocyte expression profile of severe trauma patients who develop *C.diff*. infections may confirm this hypothesis (Table 1). Increased expression of *HMGB1* and downregulation of *IRAK3* can lead to a hyperinflammatory response. Other genes crucial for an adaptive response to *C.diff*. appear to have their expression altered in a manner that is detrimental to protective immunity (as compared to non-*C.diff*.). This includes genes that encode for products involved in antigen presentation, T cell and TH17 cell differentiation as well as receptors to IgA. Thus, an individual's specific immune response to inflammation may create an environment in the host that is optimal for *C.diff*. pathogenicity. Gene expression profiles may be able to predict those individuals at risk as well as identify potential therapeutic targets.

This study has several limitations. Only 5 CDAD patients with had microarray analysis and the results require prospective validation. This study also does not demonstrate that the

transcription of specific genes equates to protein translation. Finally, gene expression technology remains expensive. Screening of all severe trauma patients will hopefully be feasible as the technology advances or the number of genes can be narrowed, allowing less expensive technology, such as multiplex mRNA determinations, to be utilized. Also, this data is specific to the trauma population, and medical and other surgical populations may have different risk factors (12).

In conclusion, blunt trauma patients who develop CDAD may be a unique population who might have specific genomic risk factors that make them susceptible to the disease. The early genomic analysis of trauma patients reveals a distinct leukocyte expression profile of *C.diff.* patients both after and prior to their infection. The latter indicates a possible association with the patient's hospital admission genomic expression profile and their susceptibility to *C.diff.* Further expression analysis of this *C.diff.* population may reveal potential therapeutic interventions and allow early identification of *C.diff.* susceptible patients.

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Figure 1. Dendrograms and Heat Maps of Supervised Analysis (*C. diff.* cluster outlined in black)
 A. 5 Patients before (β) and after (α) infection with *C. diff.* 118 genes were differentially expressed. B. 5 Patients with CDAD (1) compared to 16 age and ISS matched patients (0) without infection. 47 genes differentiated the groups. C. Comparison of 5 *C. diff.* patients (1) to 16 age and ISS matched controls (0) prior to infection. 501 genes differentiated the two groups with up to 87% class prediction.

Figure 2. Examples of Molecular Pathways from Ingenuity Analysis of *C. diff.* Patients Prior to Infection

A. Molecular pathway including genes involved in cell morphology, cellular growth and proliferation as well as cellular development. B. Molecular pathway representing genes in cell-to-cell signaling and interaction, hematological system development and function as well as immune cell trafficking.

Table 1Differentially expressed genes of interest in *C. diff.* trauma patients prior to infection.

GENE	UP vs. DOWN REGULATION	ROLE OF TRANSCRIPTION PRODUCT
<i>RFX1</i> MHC class II regulatory factor RFX1	↓	Required for MHC class II gene expression in antigen presenting cells.
<i>STAT3</i> Signal transducer and activator of transcription	↓	Essential for the differentiation of TH17 cells, which play an important role in anti-microbial immunity at epithelial/mucosal barriers.
<i>CD46</i> CD46 complement regulatory protein	↑	Inhibits complement system.
<i>HMGB1</i> High-mobility group box 1	↑	Unregulated over-expression may contribute to hyperinflammatory pathology.
<i>IRAK3</i> Interleukin-1 receptor-associated kinase 3	↓	Negatively regulates responses to Toll-like receptors and cytokine mediated signaling as well as NF-κB transcription, especially in monocytes and macrophages.
<i>IL4R</i> Interleukin 4 receptor	↓	Promotes T cell differentiation.
<i>CEBPD</i> CCAAT/enhancer-binding protein delta	↓	Regulates genes involved in immune/inflammatory responses as well as activation/differentiation of macrophages.
<i>FCAR</i> Fc Fragment of IgA receptor	↓	Receptor on leukocytes that interacts with IgA-opsinized targets, subsequently inducing inflammatory mediator release, phagocytosis, and antibody-dependent cell mediated cytotoxicity.