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Gene expression patterns in peripheral blood leukocytes in patients with recurrent ciguatera fish poisoning: Preliminary studies

Maria-Cecilia Lopez [a], Ricardo F. Ungaro [b], Henry V. Baker [a,b,c], Lyle L. Moldawer [b], Alison Robertson [d], Margaret Abbott [e], Sparkle M. Roberts [f], Lynn M. Grattan [f], J. Glenn Morris Jr. [e,g,*]

[a] Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, FL 32610, USA
[b] Department of Surgery, College of Medicine, University of Florida, Gainesville, FL 32610, USA
[c] Genetics Institute, University of Florida, Gainesville, FL 32610, USA
[d] Marine Ecotoxicology Lab, Department of Marine Sciences, University of South Alabama & Dauphin Island Sea Lab, USA
[e] Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610, USA
[f] Department of Neurology, Division of Neuropsychology, University of Maryland School of Medicine, 110 S., Paca St., 3rd Floor, Baltimore, MD 21201, USA
[g] Department of Medicine, College of Medicine, Emerging Pathogens Institute, University of Florida, 2055 Mowry Road, Box 100009, Gainesville, FL 32610, USA

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ABSTRACT

Ciguatera fish poisoning (ciguatera) is a common clinical syndrome in areas where there is dependence on tropical reef fish for food. A subset of patients develops recurrent and, in some instances, chronic symptoms, which may result in substantial disability. To identify possible biomarkers for recurrent/chronic disease, and to explore correlations with immune gene expression, peripheral blood leukocyte gene expression in 10 ciguatera patients (7 recurrent and 3 acute) from the U.S. Virgin Islands, and 5 unexposed Florida controls were evaluated. Significant differences in gene expression were noted when comparing ciguatera patients and controls; however, it was not possible to differentiate between patients with acute and recurrent disease, possibly due to the small sample sizes involved.
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1. Introduction

Ciguatera fish poisoning (CFP) is caused by consumption of tropical reef fish carrying ciguatoxin, a toxin which originates in species of the dinoflagellate *Gambierdiscus*, and is passed up through the marine food chain. Global estimates of incidence are in the range of 50,000–500,000 cases per year (Fleming et al., 1998). In studies conducted by the research team in 2011–2012 on St. Thomas, U.S. Virgin Islands, incidence was 12 cases/1000 people/year, with 41% of cases representing recurrent illness (Radke et al., 2013a).

Illness is characterized by a distinctive combination of gastrointestinal (diarrhea and/or vomiting) and neurologic (circumoral and/or extremity paresthesias, weakness and pain in legs, fatigue) symptoms; while gastrointestinal symptoms tend to resolve within 48 h, neurologic symptoms can persist in acute cases for several weeks (Morris et al., 1982a; Bagnis et al., 1979; Radke et al., 2013b). Multiple studies have found that patients who have had ciguatera once are significantly more likely to have recurrent episodes (Bagnis et al., 1979; Glaziou & Martin, 1993; Morris et al., 1982b; Radke et al., 2013a). A subset of patients develop "chronic" ciguatera, which is marked by persistence of symptoms, fatigue (reminiscent of chronic fatigue syndromes (Pearn, 1997)), and long-term disability.

There are currently no diagnostic tests for ciguatera: diagnosis is based on clinical presentation, and confirmatory electromyography, where appropriate. Factors responsible for increased rates of recurrence among persons who have had an initial episode of ciguatera are not well understood, nor is it understood why a subset of patients go on to have symptoms of chronic disease. To look for biomarkers of illness, and explore factors that may contribute to clinical presentation, gene expression in peripheral

* Corresponding author. Tel.: (352) 273-7526.
E-mail addresses: arobertson@disl.org (A. Robertson), sroberts@som.umaryland.edu (S.M. Roberts), lgrattan@som.umaryland.edu (L.M. Grattan), jgmorris@epi.ufl.edu (J.G. Morris Jr.).

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blood leukocytes (PBLs) collected from 10 patients in the U.S. Virgin Islands who had been diagnosed with CFP and 5 healthy control subjects from Florida was assessed.

2. Methods

Ten patients with CFP were drawn from a larger study of acute and recurrent CFP on the Island of St. Thomas, U.S. Virgin Islands. As previously described (Radke et al., 2013b), patients were enrolled from the Emergency Department of the Roy Lester Schneider Hospital (the only hospital on St. Thomas), with follow-ups at 3, 6, and 12 months after acute presentation. Seven of the 10 patients had been followed for between 3 and 12 months after acute illness, and had recurrent symptoms. Three patients did not have recurrent symptoms after their initial illness. For one patient, blood was drawn for the current study approximately 4 months from the time of acute illness; the remaining two patients were approximately 2 weeks from the time of their acute episode of illness, and still had residual symptoms. The mean age of ciguatera case patients was 55 years; 4 were male and 6 female. Eight of the 10 patients had an underlying medical diagnosis, including diabetes (2 patients), arthritis (2), hypertension (4), atherosclerotic heart disease (1), thyroid disease (1), and asthma (1). Samples were also collected from 5 control persons: mean age was 35; 4 were male and 1 was female; all lived in Florida; all were in good health; and none had any medical history consistent with CFP. Studies were approved by the Institutional Review Board of the University of Florida.

Whole Blood was collected into a 7.0 mL K3 EDTA vacutainer tube (Becton Dickinson #366450). The whole blood was lysed with Buffer EL (Qiagen #79217) to eliminate red blood cells and isolate the total leukocyte population. For the CFP patient samples only, RNAprotect Cell Reagent (Becton Dickinson #76526) was added to the resultant total leukocyte population to stabilize the cells for subsequent shipment to Florida. The total leukocytes for the CFP and control patients were then processed with the Qiagen RNeasy Plus Mini Kit (Qiagen #74134) to isolate the RNA.

Total RNA was quantified spectrophotometrically using the NanoDrop 1000 instrument (Thermo Scientific, Wilmington, DE). RNA quality was assessed using an RNA PicoChip on an Agilent 2100 BioAnalyzer (Agilent, Andover, MA). All specimens had RNA Integrity Number (RIN) scores greater than 5. Hundred nanograms of total RNA was processed for GeneChip analysis using the GeneChip® WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA) following manufacturer's recommendations. 5.5 μ coof resulting cDNA was fragmented, terminally labeled, and targets were hybridized to Affymetrix GeneChip® Human Transcriptome Array 2.0 (HTA 2.0) for 16 h at 45 °C and washed according to Affymetrix fluidics protocols FS450_001. Microarrays were normalized using Robust Multi-array Average (RMA) as implemented in Partek Genomics Suite 6.6 (Partek Incorporated, St Louis MO). Only annotated probe sets were used in the subsequent analysis. The resulting 26,831 annotated probe sets represented 25,193 genes. Significant genes ($p < 0.001$) were identified using the class prediction tool implemented in Biometric Research Branch BRB-ArrayTools version 4.3.0 Stable Release, developed by Richard Simon & BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). The ability of genes significant at $p < 0.001$ to distinguish between the classes was determined using leave-one-out-cross-validation studies and Monte Carlo simulations using algorithms implemented in BRB-Array Tools. Gene set analysis was also conducted using BRB ArrayTools.

3. Results and discussion

Significant differences in PBL gene expression patterns were seen in CFP patients compared with controls, with 3285 of 26,831 genes screened having a significance of $p < 0.001$. Findings are reflected in the principal component analysis shown in Fig. 1, and the heat map in Fig. 2. Significant differences in gene expression were not seen when CFP patients with recurrent symptoms were compared with those with only acute symptoms. In assessing differences in patterns of expression of genes/gene pathways, the strongest associations were with genes linked with platelet aggregation (h_ephA4Pathway) and chemokine gene expression (h_fMLPPathway) (Table 1).

The finding of significant differences in PBL gene expression patterns between patients in the U.S. Virgin Island ciguatera population and a healthy control population from Florida must be approached with caution. It is well recognized that gene expression studies have the potential to yield false positive findings if the ancestry of cases and controls are not appropriately matched, as gene expression can be both heritable and under strong genetic control (Byrnes et al., 2009; Cheung et al., 2005). In these studies, the majority of the CFP patients came either from the Virgin Islands or neighboring islands in the Caribbean; the controls, in contrast, tended to have Caucasian backgrounds. To confirm the findings, it will be necessary to conduct additional studies, with carefully matched cases and controls. Similarly, failure to find significant differences in gene expression between patients with recurrent and acute ciguatera is of uncertain relevance: acute case numbers, in particular, were small, and blood was drawn, in one instance, 4 months after the initial acute episode.

There are also uncertainties regarding the physiologic relevance of the gene pathways that were shown to have significantly different expression patterns. Ciguatoxin, the causative agent for the disease, potentiates voltage-gated sodium channels, with resultant effects on neurons; there is also a suggestion that it has direct, calcium-mediated enterotoxin activity (Fasano et al., 1991). While relevance to human cases is uncertain, there is one study of gene expression in mouse brains exposed to ciguatoxin in which there was enrichment of expression pathways related to complement and coagulation cascades (Ryan et al., 2010). Given the clinical similarities between chronic ciguatera and chronic fatigue syndromes, it could be hypothesized that similar physiologic mechanisms were operational in the two syndromes. There was

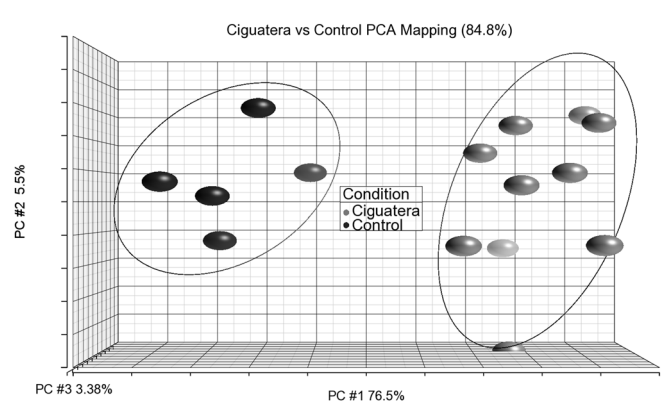

Fig. 1. A 3D principal component analysis (PCA) plot of the RNA data that characterizes the trends exhibited by the expression profiles of ciguatera fish poisoning (gray) and healthy controls (black). Each dot represents a sample and the shade represents the type of the sample.

Table 1
A list of gene sets that pass $p < 0.005$ threshold among any one of the four tests: Least Square (LS) permuation test, the Kolmogorov–Smirnov (KS) permutation test, and Efron–Tibshirani's Gene Set Analysis (GSA) maxmean test.

	Biocarta pathway	Pathway description	Number of genes	Least Square permutation *p*-value	Kolmogorov–Smirnov permutation *p*-value	Efron–Tibshirani's Gene Set Analysis test *p*-value
1	h_ephA4Pathway	**Eph Kinases and ephrins support platelet aggregation**	8	**<0.005**	**<0.005**	**<0.005 (−)**
2	h_fMLPpathway	**fMLP induced chemokine gene expression in HMC-1 cells**	34	**<0.005**	**<0.005**	**<0.005 (+)**
3	h_il7Pathway	**IL-7 Signal Transduction**	15	**<0.005**	**<0.005**	0.04 (−)
4	h_mapkPathway	**MAPKinase Signaling Pathway**	85	**<0.005**	**<0.005**	0.04 (+)
5	h_ptdinsPathway	**Phosphoinositides and their downstream targets.**	23	**<0.005**	**<0.005**	**<0.005 (+)**
6	h_keratinocytePathway	**Keratinocyte Differentiation**	57	**<0.005**	**<0.005**	0.085 (+)
7	h_pyk2Pathway	**Links between Pyk2 and Map Kinases**	27	**<0.005**	**<0.005**	0.03 (+)
8	h_bcrPathway	**BCR Signaling Pathway**	33	**<0.005**	**<0.005**	0.015 (−)
9	h_metPathway	**Signaling of Hepatocyte Growth Factor Receptor**	33	**<0.005**	**<0.005**	0.075 (+)
10	h_tcrPathway	**T Cell Receptor Signaling Pathway**	44	**<0.005**	**<0.005**	0.03 (−)
11	h_monocytePathway	**Monocyte and its Surface Molecules**	12	**<0.005**	0.009	0.025 (−)
12	h_ucalpainPathway	**uCalpain and friends in Cell spread**	14	**<0.005**	**<0.005**	0.065 (+)
13	h_lymphocytePathway	**Adhesion Molecules on Lymphocyte**	10	**<0.005**	0.007	0.01 (−)
14	h_tidPathway	**Chaperones modulate interferon Signaling Pathway**	29	**<0.005**	**<0.005**	0.08 (+)
15	h_p53hypoxiaPathway	**Hypoxia and p53 in the Cardiovascular system**	24	**<0.005**	**<0.005**	0.06 (+)
16	h_fcer1Pathway	**Fc Epsilon Receptor I Signaling in Mast Cells**	39	**<0.005**	**<0.005**	0.18 (+)
17	h_integrinPathway	**Integrin Signaling Pathway**	34	**<0.005**	**<0.005**	0.01 (+)
18	h_HivnefPathway	**HIV-I Nef: negative effector of Fas and TNF**	67	**<0.005**	**<0.005**	0.23 (+)
19	h_neutrophilPathway	**Neutrophil and Its Surface Molecules**	9	**<0.005**	0.013	0.015 (+)
20	h_il2rbPathway	**IL-2 Receptor Beta Chain in T cell Activation**	37	**<0.005**	**<0.005**	0.135 (+)
21	h_pparaPathway	**Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa(alpha)**	63	**<0.005**	0.022	0.035 (+)
22	h_bArrestin-srcPathway	**Roles of ¿-arrestin-dependent Recruitment of Src Kinases in GPCR Signaling**	17	**<0.005**	0.054	0.015 (+)
23	h_biopeptidesPathway	**Bioactive Peptide Induced Signaling Pathway**	29	**<0.005**	0.006	0.04 (+)
24	h_SARSpathway	**SARS Coronavirus Protease**	7	**<0.005**	0.022	**<0.005 (−)**
25	h_thelperPathway	**T Helper Cell Surface Molecules**	12	**<0.005**	**<0.005**	0.125 (−)
26	h_rac1Pathway	**Rac 1 cell motility signaling pathway**	21	**<0.005**	**<0.005**	0.085 (+)
27	h_hcmvPathway	**Human Cytomegalovirus and Map Kinase Pathways**	17	**<0.005**	0.025	0.045 (+)
28	h_ecmPathway	**Erk and PI-3 Kinase Are Necessary for Collagen Binding in Corneal Epithelia**	19	**<0.005**	**<0.005**	0.145 (−)
29	h_erkPathway	**Erk1/Erk2 Mapk Signaling pathway**	27	**<0.005**	0.044	0.1 (+)
30	h_no2il12Pathway	**NO2-dependent IL 12 Pathway in NK cells**	15	**<0.005**	0.015	0.08 (−)
31	h_dcPathway	**Dendritic cells in regulating TH1 and TH2 Development**	17	**<0.005**	0.149	0.2 (−)
32	h_nkcellsPathway	**Ras-Independent pathway in NK cell-mediated cytotoxicity**	27	**<0.005**	0.070	0.2 (−)
33	h_ceramidePathway	**Ceramide Signaling Pathway**	21	**<0.005**	0.140	0.05 (+)
34	h_stat3Pathway	**Stat3 Signaling Pathway**	8	**<0.005**	**<0.005**	0.05 (+)
35	h_chemicalPathway	**Apoptotic Signaling in Response to DNA Damage**	18	**<0.005**	0.057	0.105 (−)
36	h_rasPathway	**Ras Signaling Pathway**	22	**<0.005**	**<0.005**	0.015 (+)
37	h_agrPathway	**Agrin in Postsynaptic Differentiation**	35	**<0.005**	0.087	0.175 (+)
38	h_At1rPathway	**Angiotensin II mediated activation of JNK Pathway via Pyk2 dependent signaling**	27	**<0.005**	0.015	0.045 (+)
39	h_crebPathway	**Transcription factor CREB and its extracellular signals**	22	**<0.005**	0.006	0.07 (+)
40	h_cxcr4Pathway	**CXCR4 Signaling Pathway**	19	**<0.005**	**<0.005**	0.055 (+)
41	h_stathminPathway	**Stathmin and breast cancer resistance to antimicrotubule agents**	20	**<0.005**	**<0.005**	0.12 (−)
42	h_calcineurinPathway	**Effects of calcineurin in Keratinocyte Differentiation**	18	**<0.005**	**<0.005**	0.06 (−)
43	h_tcytotoxicPathway	**T Cytotoxic Cell Surface Molecules**	12	**<0.005**	**<0.005**	0.135 (−)
44	h_erk5Pathway	**Role of Erk5 in Neuronal Survival**	14	**<0.005**	**<0.005**	0.025 (+)
45	h_fasPathway	**FAS signaling pathway (CD95)**	34	**<0.005**	0.234	0.225 (−)
46	h_ghPathway	**Growth Hormone Signaling Pathway**	27	**<0.005**	**<0.005**	0.035 (+)
47	h_d4gdiPathway	**D4-GDI Signaling Pathway**	12	**<0.005**	0.012	0.21 (−)
48	h_RacCycDPathway	**Influence of Ras and Rho proteins on G1 to S Transition**	26	**<0.005**	0.123	0.03 (+)
49	h_ptenPathway	**PTEN dependent cell cycle arrest and apoptosis**	18	**<0.005**	**<0.005**	0.055 (+)
50	h_tcapoptosisPathway	**HIV Induced T Cell Apoptosis**	9	0.008	**<0.005**	0.14 (−)
51	h_malPathway	**Role of MAL in Rho-Mediated Activation of SRF**	19	0.008	**<0.005**	0.01 (+)
52	h_ps1Pathway	**Presenilin action in Notch and Wnt signaling**	14	0.009	**<0.005**	0.08 (+)
53	h_mef2dPathway	**Role of MEF2D in T-cell Apoptosis**	17	0.009	**<0.005**	0.01 (−)
54	h_hdacPathway	**Control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK)**	25	0.017	**<0.005**	0.235 (−)
55	h_RELAPathway	**Acetylation and Deacetylation of RelA in The Nucleus**	21	0.017	**<0.005**	0.125 (+)
56	h_ranbp2Pathway	**Sumoylation by RanBP2 Regulates Transcriptional Repression**	11	0.019	**<0.005**	0.07 (−)
57	h_salmonellaPathway	**How does salmonella hijack a cell**	5	0.049	**<0.005**	0.15 (−)
58	h_pitx2Pathway	**Multi-step Regulation of Transcription by Pitx2**	15	0.059	**<0.005**	0.14 (+)
59	h_ctla4Pathway	**The Co-Stimulatory Signal During T-cell Activation**	30	0.062	**<0.005**	0.255 (−)
60	h_classicPathway	**Classical Complement Pathway**	27	0.779	**<0.005**	0.515 (+)
61	h_compPathway	**Complement Pathway**	38	0.800	**<0.005**	0.465 (+)

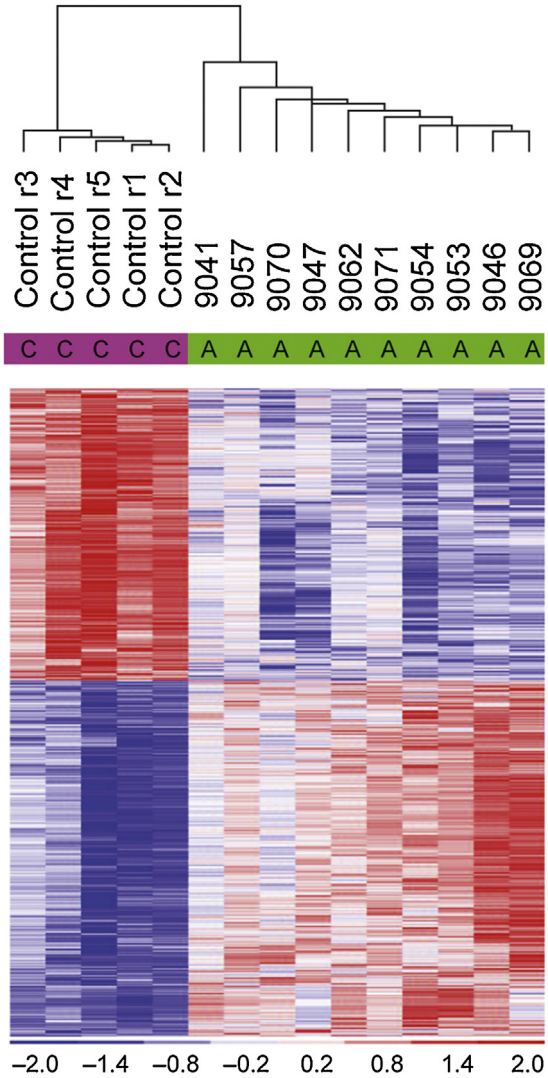

Fig. 2. Hierarchical clustering of 3285 genes differentially expressed between CFP and controls at a significance of $p < 0.001$. Each row of the data matrix represents a gene and each column represents a sample. Expression levels are depicted according to the color scale shown at the bottom. Red and blue indicate expression levels respectively above and below the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

some overlap with gene pathways identified in patients with chronic fatigue (e.g., ARF1 (Vernon et al., 2002); CEACAM family (Kaushik et al., 2005); however, more recent studies have raised questions about the relevance of these findings (Byrnes et al., 2009).

Ciguatera remains an important clinical entity in areas where there is high dependence on tropical reef fish for food. Identification of a biomarker for CFP (particularly recurrent or chronic CFP), and/or for CFP susceptibility, would have substantial clinical relevance, both in terms of diagnosis, and as a basis for development of therapeutic interventions. Findings of this preliminary study are intriguing: there is a need to follow-up on these results (with appropriate control populations) and to further explore potential physiologic relevance of the identified gene pathways.

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