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Research Report

Correlation of psycho-neuroendocrine-immune (PNI) gene expression with symptoms of acute infectious mononucleosis

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ARTICLE INFO

Article history:
Accepted 6 November 2005
Available online 25 December 2005

Theme:
Cellular and molecular biology
Topic:
Gene structure and function: general

Keywords:
Psycho-neuroendocrine-immune (PNI)
Microarray
Infectious mononucleosis
Gene expression
Epstein–Barr virus (EBV)
MEF2C
HCRTR2
VACHT

ABSTRACT

Acute infection is known to perturb psycho-neuroendocrine-immune (PNI) gene expression. Oligonucleotide microarrays were used to examine PNI gene expression in the peripheral blood of 13 subjects with infectious mononucleosis (IM). Novel peripheral blood gene expression activity was correlated with central-nervous-system-mediated symptoms including fatigue and sleep disturbance. Of note, expression of the MADS box transcription enhancer factor 2 polypeptide C (*MEF2C*) gene, previously implicated in skeletal muscle myogenesis, correlated with symptoms of musculo-skeletal pain and fatigue. Expression of the hypocretin/orexin receptor *HCRTR2*, which has been implicated in narcolepsy, correlated with sleep disturbance. And, *VACHT*, the vesicular acetylcholine transporter, was highly correlated with neurocognitive disturbance. The expression of both *HCRTR2* and *MEF2C* in the peripheral blood was validated by reverse transcription PCR. Thus, investigation of the PNI response in peripheral blood may provide novel insights into the complex pathophysiology of centrally mediated disease states.

Published by Elsevier B.V.

1. Introduction

The recent discovery that approximately 65% of 1600 genes that mediate psychological, neuroendocrine, and immune (PNI) processes are expressed in peripheral blood mononuclear cells (PBMCs) (Nicholson et al., 2004) suggests novel mechanisms for communication between the immune system and the brain. Expression of a surprising number of these genes has only been described previously within the nervous and endocrine tissues. It is now possible to use circulating blood as a molecular biopsy and alternative to tissue-specific gene expression to identify peripheral markers that decipher centrally mediated disorders (Tsuang et al., 2005; Segman et al., 2005).

Peripheral blood gene expression during recovery from acute infection was evaluated as a tool for investigating

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0006-8993/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.brainres.2005.11.013

responses mediated by the CNS. The acute sickness response following infection exemplifies how the brain and the periphery choreograph response to an insult with the intention of returning the body to homeostasis. Acute infection typically manifests with a cluster of symptoms that includes fever, an increased need to sleep, musculo-skeletal pain, impaired concentration, irritability or depressed mood, anorexia, and a loss of interest in usual activities, social interaction, and body care (Dantzer, 2001; Hart, 1988). These symptoms were recorded, and the peripheral blood of 13 people with infectious mononucleosis (IM) following documented infection with Epstein–Barr virus (EBV) was examined using microarrays. Symptoms were correlated with gene expression levels. Genes known to be associated with myogenesis, acetylcholine transport and narcolepsy were expressed in the peripheral blood and highly correlated with symptoms of acute sickness including pain, neurocognitive and sleep disturbance.

2. Results

The demographic characteristics and symptom scores of the 13 subjects are detailed in Table 1. Most subjects were in their late teenage or early adulthood years, which is typical of symptomatic IM resulting from acute EBV infection. The reported symptoms were characteristic of acute sickness in general and of IM in particular. The most commonly reported symptom was malaise (13/13) followed by fever, neurocognitive difficulties, and sleep disturbance (12/13), fatigue (11/13), irritability/depression (9/13), and muscle or joint pain (7/13). Of interest, individual subjects reported quite varied symptom scores for each of the seven components. For example, scores ranging from 0 to 5 were found for symptom elements within individual subjects, and scores ranging from 0 to 5 were also evident when comparing responses for an individual symptom between subjects (Table 1).

To determine gene expression correlates of acute infection, 1058 PNI genes were correlated with the seven symptom scores. Three analyses were performed, with generally convergent results. Both Spearman Rho and class prediction correlations were determined iteratively, using a leave-one-out and permutation analysis. There were 18 PNI genes that were significantly correlated with coefficients between 0.7 and −0.7 with one or more of the symptom dimensions (Table 2). Of these, 3 (17%) were primarily involved in endocrine functions, 8 (44%) were immune system genes, 3 (17%) had well-documented neuronal roles, and 4 (22%) had global or multi-system roles.

A stepwise multiple linear regression was performed for each symptom dimension to identify a subset of genes that could explain most of the variability in symptom scores between individuals. As this technique is hierarchical, each gene selected by this statistical process has been prioritized above other candidates and therefore may represent an independent biological pathway. In all cases, three or four PNI genes accounted for >90% of the variance in the gene expression associated with that symptom. These genes are diagrammed in Fig. 1. The three main correlates of sleep disturbance (*HCRTR2*, *FCGR2B*, and *FOXA3*) by the stepwise linear regression were also found to be consistently and highly correlated by both Spearman Rho and class prediction analysis. The single gene correlate of fatigue found using the Spearman Rho and class prediction, *MEF2C*, accounted for 62% of the variance by stepwise linear regression. Two gene correlates of muscle/joint pain, *MEF2C* and *FLJ12541*, accounted for 84% of the variance by stepwise linear regression. For neurocognitive disturbance, *CD3G* accounted for 58% of the variance. The remaining three symptoms (fever, malaise, and irritability/depression) had no overlap in results with the other statistical methods.

To validate the correlation between genes and symptoms, we took advantage of the fact that half the subjects had high fatigue scores and searched for differential gene expression based on fatigue severity. Seven of the 13 subjects had high fatigue scores of 4 and 5, and the remaining 6 subjects had low fatigue scores of 0, 1 or 2. Using a two-tailed *t* test, all 1058 genes were compared between the 7 high fatigue subjects and the 6 low fatigue subjects. Importantly, only *MEF2C*, the same gene identified by all statistical tests, was significantly differentially expressed between the high and low fatigue groups ($P = 0.0015$).

Table 1 – Age, sex, weeks post-onset, and symptom scores for each subject

Age	Sex	Weeks post-onset	Symptom Scores						
			Fatigue	Fever	Irritability/ depression	Malaise	Muscle/joint pain	Neurocognitive disturbance	Sleep disturbance
18	F	3	0	2	0	1	1	1	1
19	M	4	5	4	5	2	3	4	3
17	F	3	5	3	5	3	4	4	3
17	M	8	4	4	5	3	2	3	2
23	F	4	5	2	5	2	4	2	5
18	F	2	2	4	0	3	0	1	1
49	F	5	4	4	2	3	5	4	5
18	F	5	2	0	5	4	0	2	5
18	M	5	5	5	5	4	4	3	3
34	F	4	1	2	1	3	0	3	3
18	M	3	0	3	0	3	0	0	0
19	F	4	1	3	2	2	0	2	2
48	M	3	4	4	0	2	0	1	1

Table 2 – Correlations between symptoms and PNI gene expression in 13 subjects with IM

Gene ID, gene name	Fatigue	Fever	Irritability/ depression	Malaise	Muscle/ joint pain	Neurocognitive disturbance	Sleep disturbance
FCGR2B, Fc fragment of IgG, low affinity IIb, receptor for (CD32)	0.26	0.04	0.64	0.57	0.34	0.61	**0.74**
HCRTR2, hypocretin (orexin) receptor 2	0.33	−0.22	0.34	0.06	0.43	0.48	**0.75**
NCOA1, nuclear receptor coactivator 1	0.3	−0.08	0.47	0.54	0.32	0.58	**0.74**
TNFRSF10B, tumor necrosis factor receptor superfamily, member 10b	0.19	−0.24	0.44	0.3	0.3	0.58	**0.71**
FOXA3, forkhead box A3	0.54	−0.02	0.62	0.03	0.62	0.42	**0.8**
SCAMP2, secretory carrier membrane protein 2	0.54	0.14	0.6	0.32	**0.72**	0.68	**0.79**
FLJ12541, stimulated by retinoic acid gene 6	0.27	0.14	0.35	0.05	**0.73**	0.55	0.36
HSD17B3, hydroxysteroid (17-beta) dehydrogenase 3	0.3	−0.07	0.29	−0.16	**0.7**	0.62	0.58
NRG2, neuregulin 2	0.39	0.18	0.43	0.04	**0.75**	0.49	0.3
MEF2C, MADS box transcription enhancer factor 2, polypeptide C	**0.85**	0.42	0.6	0.1	**0.81**	**0.77**	0.63
BTK, Bruton agammaglobulinemia tyrosine kinase	0.28	−0.09	0.57	0.43	0.18	**0.74**	0.68
CD3G, CD3G antigen, gamma polypeptide (TiT3 complex)	0.28	0.19	0.31	0.09	0.36	**0.79**	0.26
VACHT, vesicular acetylcholine transporter	0.34	0.04	0.47	0.24	0.68	**0.77**	0.55
ITGA4, integrin, alpha 4 (CD49D, alpha 4 subunit of VLA-4 receptor)	0.2	0.06	0.5	0.16	0.39	**0.74**	0.47
CDC37, CDC37 cell division cycle 37 homolog (*S. cerevisiae*)	0.07	0.21	0.15	**0.78**	0.21	0.25	0.28
NSEP1, nuclease sensitive element binding protein 1	0.26	**0.72**	0	−0.11	0.33	−0.04	−0.21
PMCH, pro-melanin-concentrating hormone	0.43	−0.41	**0.76**	0.2	0.4	0.35	0.55
PTPN6, protein tyrosine phosphatase, non-receptor type 6	0.47	0.03	**0.73**	0.23	0.3	0.39	0.34

Significant correlations are noted in bold.

Because expression of *HCRTR2* has not been extensively characterized in tissues other than the brain, we used LightCycler-based real-time RT-PCR to validate its expression in addition to *MEF2C*, which is known to be transcribed in the blood. The expression of both *HCRTR2* and *MEF2C* was detected in PBMC total RNA of all 9 subjects tested. The *MEF2C* primers resulted in a single expected product (123 bp). The *HCRTR2* primers resulted in multiple bands ranging in sizes from 150 to 500 bp (expected 285 bp only). The expected 285 bp *HCRTR2* product was present in both the peripheral blood samples and in the positive control hypothalamus total RNA. Because of the complexity in the banding pattern of *HCRTR2* gene in the peripheral blood, relative gene expression was quantified only for *MEF2C*. The mean crossing point (Cp) values of *MEF2C* (target) and *PPIB* (reference) genes were 26.18 (±0.99) and 24.40 (±0.99) respectively. There were 2 subjects, one in the low fatigue/pain group and one in the high fatigue/pain group, with expression values that were outliers in their respective group. Because of the small sample size and these outliers, we did not attempt to correlate quantitative *MEF2C* with fatigue and pain symptom scores. For the 9 samples, the relative expression of *MEF2C* normalized to *PPIB* and calibrated

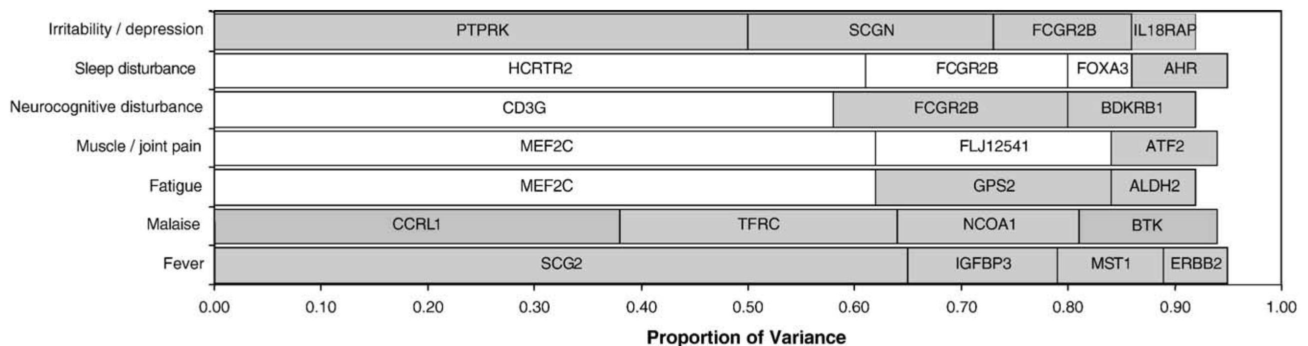

Fig. 1 – Genes influencing the severity of each symptom, and the amount of variance accounted for by stepwise linear regression following inclusion of that gene into the model. Open bars represent genes that were also found to be correlated with the symptom using the non-parametric correlation analysis.

to Stratagene reference total RNA ranged from 4- to 103-fold in the PBMCs.

3. Discussion

This analysis of gene expression patterns in the peripheral blood of subjects with acute sickness due to IM supports the potential of this approach to inform our understanding of PNI processes. The traditional notion of the biological basis of the acute sickness response is that symptoms are generated by the action of pro-inflammatory cytokines upon CNS targets, although the detailed pathways by which these immunological signals are translated into neural transmissions remain largely uncharacterized (Vollmer-Conna, 2001). The findings presented here suggest the identity of additional proteins contributing to the genesis of symptoms in acute sickness. These may be downstream elements in cytokine-triggered pathways or they may represent previously unexplored determinants of the acute sickness response.

The discovery that increased levels of mRNA encoding the hypocretin (orexin) receptor 2 (*HCRTR2*) was significantly correlated with sleep disturbance by all statistical methods and that the expression of this gene was validated in PBMCs is interesting. This gene was first described when defects in its canine homolog were discovered to cause narcolepsy (Lin et al., 1999). Expression of *HCTR2* has been extensively characterized in the brain (Greco and Shiromani, 2001; Lin et al., 2002; van den Pol et al., 2001) but only recently observed in blood (Steidl et al., 2004), where its role has not yet been ascertained. Ligand binding to neuronal hypocretin receptors promotes wakefulness, apparently by stimulating histamine release (Huang et al., 2001; Mochizuki and Scammell, 2003; Yamanaka et al., 2002). A mechanism by which increased concentrations of hypocretin receptors in cells found in peripheral blood might cause (or be the result of) sleep disturbance cannot be determined solely on the basis of this study. However, since there were multiple *HCRTR2* gene products in the peripheral blood compared to predominantly one band in the hypothalamus RNA, the expression of this gene may be differentially regulated in PBMCs. Further studies examining the correlations between regulation of hypocretin mRNA expression in leukocytes and neurons are warranted.

The major gene expression correlate of both fatigue and muscle/joint pain was found to be the MADS box transcription enhancer factor 2, polypeptide C (*MEF2C*). Expression of this gene was also validated in all peripheral blood samples tested. The MADS box is a highly conserved transcription factor sequence motif named using initials of these four originally identified members, which were the yeast MCM1 (MiniChromosome Maintenance), two floral homeotic genes AG (AGAMOUS) and DEFA (DEFICIENS), and the human *SRF* (serum response factor) (Ma et al., 1991). The protein encoded by the *MEF2C* gene is one of four variants of the transcription factor MEF2 (Leifer et al., 1993; McDermott et al., 1993), which is required for skeletal muscle myogenesis (Dodou et al., 2003) and brain development (Leifer et al., 1994; Leifer et al., 1997). *MEF2C* expression has also been found in mast cells (Wei et al., 2003) and B cells, along with another MEF2 protein, MEF2D (Swanson et al., 1998). The MEF2 transcription factor is also likely to be an important component of the host response to EBV infection as it binds to the promoter of the viral protein, BZLF1, which is a transcription factor required for expression of the remaining viral genes. During the latent phase of EBV infection, MEF2 binds HDACII, thus inhibiting transcription of BZLF1, while several inducers of viral reactivation act by converting MEF2 into a form capable of interacting with other cellular transcription factors to allow expression of BZLF1 (Liu et al., 1997; Gruffat et al., 2002). The findings presented here therefore suggest a role for the protein encoded by *MEF2C*, perhaps as a transcriptional activator of EBV gene expression during the replicative stage of infection or, conversely, as an immune response encouraging the virus towards latency. It is important to note that *MEF2C* was differentially expressed between people with a high fatigue score and those with a low fatigue score.

Neurocognitive disturbance was highly correlated with expression of *VACHT*, the vesicular acetylcholine transporter. *VACHT* is encoded by an uninterrupted sequence in the first intron of the choline acetyltransferase gene and that mRNA expression of *VACHT* colocalized with choline acetyltransferase (Erickson et al., 1994). And, the expression of choline acetyltransferase has been characterized in many non-neuronal tissues including peripheral blood cells (Wessler et al., 1998). Memory and cognitive deficits are closely associated with dysfunction of central cholinergic system. And, non-neuronal choline acetyltransferase is involved in multiple cell functions such as mitosis, migration, signaling, and immune function. How peripheral choline acetyltransferase expression affect central nervous system functions such as memory and cognition remains to be elucidated.

Much remains unknown about the full spectrum of contributors to the symptom complex of acute sickness and the details of how the immune signals are ultimately translated into changes in neural transmission. It is intuitively appealing to think that the same pathways, regardless of etiology, generate symptoms of acute infection. The data presented here raise the possibility that individual components of the acute sickness response may involve multiple additional pathways following initiation of the symptom complex. However, gene expression correlates identified here may be specific to EBV infection and may relate more directly to the host response to EBV rather than to the genesis of symptoms. Despite limitations of statistical power imposed by its relatively small sample size, this pilot study demonstrates that analysis of gene expression in peripheral blood can be a valuable tool for investigating PNI responses.

4. Experimental procedures

4.1. Samples

Peripheral blood samples were available from subjects in an ongoing prospective cohort study (the Dubbo Infection Outcomes Study) based in the township of Dubbo in rural New South Wales, Australia (Bennett et al., 1998). Subjects were enrolled following presentation to their general practitioner with symptoms suggestive of IM and laboratory documentation of IgM antibodies against Epstein–Barr virus (EBV) viral capsid antigen (VCA) (Robertson et al., 2003). Samples were obtained 3–8 weeks

following onset of symptoms ($n = 13$). The study protocol was approved by the appropriate human research ethics institutional review boards. Written informed consent was provided by all subjects.

4.2. Symptom assessment

The pattern and severity of symptoms were recorded using a 34-item self-report questionnaire—the Somatic and Psychological Health Report (SPHERE) (Hadzi-Pavlovic et al., 2000; Hickie et al., 2001), the Profile of Mood States (POMS) (McNair et al., 1971), and a 17-item checklist of other physical symptoms, such as rash and night sweats, that are relevant to IM. Selected questions were specific to the self-reported experience of fever ("had a fever," "been having chills"), malaise ("generally felt unwell," "been feeling listless"), fatigue ("prolonged tiredness," "tired after rest"), muscle/joint pain ("had muscle aches and pains," "had pains in joints"), neurocognitive disturbance (poor concentration; "feeling lost for the word"), sleep disturbance ("needing to sleep longer," "waking up tired"), and irritability/depression ("getting annoyed easily," "feeling unhappy or depressed"). From these items, a seven-component set of symptom scores for each subject was generated, each being a composite of dichotomous scores (0, 1) from responses to five items, generating a possible range of scores for each symptom domain of 0–5.

4.3. Gene expression profiling

Peripheral blood samples were transported from the field under controlled temperatures (15–25 °C) and processed within 6 h of collection. Blood collection and handling employed strict endotoxin-minimized conditions. PBMCs were separated by density gradient centrifugation (Lymphoprep; AXIS-SHIELD, Norway), cryopreserved in RPMI (Trace, Australia) with 10% DMSO (Sigma, Australia) and 50% autologous plasma, and aliquots were stored in the vapor phase of liquid nitrogen. Total RNA was subsequently extracted by use of TRIZOL (Invitrogen, Carlsbad, CA), and the quality was assessed by gel electrophoresis. Total RNA was labeled, hybridized to the 10,000 feature MWG-A array (MWG Biotech, Inc., Highpoint, NC) and microarray images captured and analyzed as described previously (Ojaniemi et al., 2003).

4.4. Real-time RT-PCR

LightCycler-based real-time RT-PCR (Rajeevan et al., 2001) using SYBRgreen dye I detection and melting curve analysis for product specificity was used for the determination of relative expression of MEF2C and HCRTR2 (target genes) in 9 samples with remaining RNA. One microgram total RNA was reverse transcribed using oligo-dT and random primers and Powerscript reverse transcriptase (Clontech, CA) in 20 μl of cDNA synthesis reaction. Based on previous report (Pachot et al., 2004), we used the gene PPIB (peptidylpropyl isomerase B) as the reference gene for normalization of the relative gene expression. Reference total RNA (Stratagene, CA) and human hypothalamus total RNA (Ambion, TX) were used as the calibrator samples for MEF2C and HCRTR2 genes, respectively. Two-fold serial dilutions of cDNA ranging from 8.0 to 0.5 ng of the respective calibrator samples were used for determining the efficiencies of MEF2C and HCRTR2 primers. PPIB primer efficiency was determined with both calibrator samples. Primers for target and reference genes were as follows: MEF2C primers, GAG GCG GGG AGA TCT CCT GTT GAC (fw) and GTC CGG CGA AGG TCT GGT GAG TC (rv); HCRTR2 primers; GAT GAG CGC TGG GGT GGT GA (fw) and ATC CGG GCT GTT TTC CTT CTG G (rv); and PPIB primers, AAG GGG CCC AAA GTC ACC GTC AAG (fw) and GGG GAA GCG CTC ACC GTA GAT GC (rv). A 4-segment amplification cycle and 3-segment melting cycle were used as reported previously (Rajeevan et al., 2001) except that annealing and signal acquisition temperatures were as follows: MEF2C, annealing at 58 °C and signal acquisition at 86 °C; HCRTR2, annealing at 60 °C and signal acquisition at 86 °C; PPIB, annealing at 65 °C and signal acquisition at 82 °C.

Four nanograms of cDNA from each sample was run in duplicate in LightCycler 2.0 separately for the target and reference genes. Fold change in gene expression was determined by exporting and analyzing the standard curves and sample runs for both target and reference genes into the Relative Quantification Monocolor Analysis module of the LightCycler software Version 4.0. Fold changes are calibrator-normalized relative levels with PCR efficiency correction.

4.5. Statistical analysis

For this study, we were interested in PNI genes only. There were 1182 of the 1622 PNI genes represented by one or more of the approximately 10,000 probes on the MWG-A array (Nicholson et al., 2004). The intensities for these 1182 probes were median-centered and $\log_2$-transformed. We identified, correlated, and differentially expressed genes using four statistical tests: Spearman Rho, class prediction, stepwise multiple linear regression, and t test. Spearman Rho, linear regression, and t test results were performed using JMP 5.1 statistical software (SAS Institute, Cary, NC). Class prediction was performed using BRB ArrayTools Version 3.3. Correlations between expression of 1058 PNI genes and symptoms from all 13 subjects were evaluated. A relatively conservative Spearman Rho correlation of ≥ 0.7 was considered significant (equivalent to $P < 0.007$ with $n = 13$). Stepwise multiple linear regression with a mixed entry method was performed for each symptom on the subset of PNI genes with P value ≤ 0.25, using a Pearson correlation P value for entry set at 0.25 and for exit set at 0.15. The significance of differentially expressed genes between groups based on symptom severity was identified using the t test. A multivariate permutation test with 80% confidence that the false discovery rate was less than 10% was used to compute the significance of each gene/symptom correlation. Multivariate permutations test was computed based on 1000 random permutations. Permutation P values for significant genes were computed based on 10,000 random permutations.

Finally, a reliability score for each significant gene/symptom pair was calculated. We reasoned that a spot with a low signal-to-noise (S/N) ratio that was reproducibly correlated with a given symptom could be considered more reliable than one that was not reproducibly correlated. Furthermore, a spot that had a high S/N ratio but is greatly dependent on certain individuals in the sample for a high correlation was less reliable. This reliability score was calculated as: $\log_{10}(|(\log_2$ (median of S/N) / (CV of leave-one-out Spearman Rho scores)$| * 100)$. The resulting score was linear, with a limited range (−3 to 3) and a slightly skewed, non-normal distribution. Examination of this distribution suggested that correlations scoring <0.5 were outliers resulting in approximately 30% of correlations being rejected.

Acknowledgments

We thank Rosane Nisenbaum for informative discussions regarding statistical methods. The Dubbo Infection Outcomes Study is funded by project grants from the National Health and Medical Research Council of Australia (numbers 157092 and 157062) and by a cooperative research agreement with the Centers for Disease Control and Prevention, USA (number U50/CCU019851-01).

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