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Research report

Cytotoxic lymphocyte microRNAs as prospective biomarkers for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis

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ABSTRACT

Background: Immune dysfunction associated with a disease often has a molecular basis. A novel group of molecules known as microRNAs (miRNAs) have been associated with suppression of translational processes involved in cellular development and proliferation, protein secretion, apoptosis, immune function and inflammatory processes. MicroRNAs may be implicated in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME), where immune function is impaired. The objective of this study was to determine the association between miRNAs in cytotoxic cells and CFS/ME.
Methods: Natural Killer (NK) and $CD8^+$T cells were preferentially isolated from peripheral blood mononuclear cells from all participants (CFS/ME, n = 28; mean age = 41.8 ± 9.6 years and controls, n = 28; mean age = 45.3 ± 11.7 years), via negative cell enrichment. Following total RNA extraction and subsequent synthesis of cDNA, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression levels of nineteen miRNAs.
Results: There was a significant reduction in the expression levels of *miR-21*, in both the NK and $CD8^+$T cells in the CFS/ME sufferers. Additionally, the expression of *miR-17-5p*, *miR-10a*, *miR-103*, *miR-152*, *miR-146a*, *miR-106*, *miR-223* and *miR-191* was significantly decreased in NK cells of CFS/ME patients in comparison to the non-fatigued controls.
Limitations: The results from these investigations are not yet transferable into the clinical setting, further validatory studies are now required.
Conclusions: Collectively these miRNAs have been associated with apoptosis, cell cycle, development and immune function. Changes in miRNAs in cytotoxic cells may reduce the functional capacity of these cells and disrupt effective cytotoxic activity along with other immune functions in CFS/ME patients.

1. Introduction

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a multi-symptom, multi-factorial and heterogeneous disorder. CFS/ME affects about 1–4% of individuals worldwide and is characterised by deficits in short term memory and concentration, tender lymph nodes, muscle pain, severe headaches, sleep disturbances, profound fatigue and postexertional malaise (Fukuda et al., 1994). Immune markers have been identified including decreased cytotoxic activity (Brenu et al., 2010; Klimas et al., 1990; Maher et al., 2005). Differential expressions of genes involved in immunological, neurological

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and metabolic processes have also been implicated in CFS/ME (Kaushik et al., 2005; Kerr, 2008; Kerr et al., 2008a, 2008b; Lin and Hsu, 2009; Saiki et al., 2008). A number of these genes encode transcription factors known to regulate immune function such as cytotoxicity, cytokine secretion and apoptosis and have been shown to be decreased in CFS/ME patients (Brenu et al., 2012; Faria and Weiner, 2006; Matsuoka and Jeang, 2005; Schaefer et al., 2007).

Other regulatory molecules have been identified that may have characteristics similar to transcription factors, these molecules are known as microRNAs (miRNAs). It is predicted that the human genome may encode over 1000 miRNAs (Griffiths-Jones, 2010). MicroRNAs are highly conserved non-coding RNA molecules 18–24 nucleotides in length that preferentially target 3′ untranslated regions of their target mRNAs (Sun et al., 2010). MicroRNAs are endogenously expressed and transcribed into a primary miRNA from introns of protein-/non-coding sequences of exclusive miRNA genes or host genes (Sun et al., 2010). The pri-miRNA formed is processed into a pre-miRNA and integrated into an RNA-Induced Silencing Complex (RISC) (Hammond et al., 2000). The miRNA-RISC either cleavages complementary mRNA molecules or inhibits protein translation resulting in the decrease of de novo synthesis of the corresponding protein (Behm-Ansmant et al., 2006). The interactions between miRNAs and mRNAs are important in maintaining coherent physiological processes such as immune function.

MicroRNAs are required during development, maturation, proliferation, antigen recognition, apoptosis induction and cytokine secretion of immune cells (Liston et al., 2010; O'Connell et al., 2007; Taganov et al., 2006; Tili et al., 2007). Dysregulation in the expression of miRNAs may adversely affect immune homeostasis. For example, deficiencies in *miR-155* encourage a shift towards T helper 2 (Th2) anti-inflammatory immune responses (Rodriguez et al., 2007; Thai et al., 2007), whilst in the absence of *miR-101* autoreactive T cell mediated autoimmunity occurs (Yu et al., 2007). MicroRNAs are also essential for modulating immune responses to bacterial and viral infection. Alterations in these miRNAs may significantly affect immune reactions such as cytotoxic activity which are known to be compromised in CFS/ME.

The purpose of this study was to assess the possible role of miRNAs in cytotoxic cells of the innate (NK cells) and adaptive ($CD8^+$T cells) immune system in CFS/ME patients. We hypothesised that as miRNAs can either increase or decrease the expression of various genes, they may also be involved in the regulation of cytotoxic cells in CFS/ME patients. To the best of our knowledge this is the first study to explore the role of miRNAs in cytotoxic cells of CFS/ME patients.

2. Methods

2.1. Subject recruitment

This study was approved by the Bond University Human Research Ethics Committee (R0852A). Participants ($n = 56$) for the study were recruited from a database of patients from the South East Queensland region of Australia. The inclusion criteria for CFS/ME ($n = 28$; age $= 42.0 \pm 9.4$ years) was based on the Centers for Disease Control and Prevention (CDC) 1994 case definition whilst the non-fatigued healthy control ($n = 28$; age $= 45.0 \pm 14.0$ years) were participants with no medical history or symptoms of prolonged fatigue or illness of any kind (Fukuda et al., 1994).

2.2. Sample collection and cell isolation

Venous blood samples (40 mL) from all participants were collected into EDTA tubes and analysed within 3 h of collection. Peripheral blood mononuclear cells were isolated from 20 mL of whole blood for each cell type using Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). Enrichment of NK or $CD8^+$T cells was performed using NK and $CD8^+$T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched NK or $CD8^+$T cell purity was examined on the FACSCalibur flow cytometer (BD Bioscience, San Diego, CA) after staining with CD16/CD56 or CD8/CD3 monoclonal antibodies (BD Bioscience, San Diego, CA). Flow cytometry and haemocytometer assessment were used to determine the purity of the cells isolated for miRNA expression analysis. The recovery of isolated cells was calculated based on the observation that NK and $CD8^+$T cells represent 2% and 5% of peripheral blood lymphocytes respectively (Banerjee et al., 2005; Dorfman and Raulet, 1998), hence, there are more $CD8^+$T cells in circulation compared to NK cells. Thus recovery was defined as the ratio of percentage of the total number of cells (i.e. NK or $CD8^+$T cells) isolated to the percentage of cells (i.e. NK or $CD8^+$T cells) present in the volume of blood collected. Enriched cells were snap frozen in liquid nitrogen and stored at −80 °C until further assessment.

2.3. RNA extraction and cDNA synthesis of NK and $CD8^+$T cells

Total RNA (containing miRNA) was extracted from isolated NK and $CD8^+$T cells using the miRNeasy isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Concentration and purity of RNA were determined using the NanoDrop 3300 (Thermo Scientific, Waltham, MA). Synthesis of cDNA from 250 ng of miRNA was performed using the NCode™ miRNA First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Synthesised cDNA were diluted 1:20 and stored at −20 °C prior to RT-qPCR.

2.4. Analysis of miRNA gene expression

A panel of 19 miRNAs expressed in NK and $CD8^+$T cells was selected based on their involvement in immune cell function (Table 1). Six small non-coding RNA genes (*SNORD25*, *SCARNA17*, *SNORA73A*, *RNU5A*, *RNU1A* and *RNU6B*) (Qiagen, Hilden, Germany) were assessed using GeNorm to determine their usability as reference genes (Etschmann et al., 2006). *RNU1A* was found to be the most stable and therefore served as the endogenous reference control for all miRNAs assessed in this study. RT-qPCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The final reaction volume (10 μL) included 1× iQ SYBR-Green Supermix (Bio-Rad, Hercules, CA), 200 nM of each primer and 4 μL of diluted cDNA. Primer