RGC-32, FasL, and SIRT1 as Potential Biomarkers of Relapse and Response to Treatment with Glatiramer Acetate in Multiple Sclerosis

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Background

Patients with Relapsing-Remitting Multiple Sclerosis (RRMS) are commonly managed with first-line drug treatments glatiramer acetate or interferon beta to reduce but not eliminate the frequency of relapse events over time. However, due to the heterogeneous nature of RRMS it is difficult to predict patient prognosis and response to treatment. Currently there is a critical need for the development of reliable biomarkers to aid clinicians in the management of RRMS patients. We have previously shown that the Response Gene to Complement (RGC)-32 is expressed by $CD3^+$ as well as $CD4^+$ T cells in peripheral blood mononuclear cells (PBMCs) and in brain tissue from RRMS patients [1]. We have also found that RGC-32 mRNA expression is significantly lower in patients with relapses compared to those in remission and healthy controls [1]. RGC-32 is a recently identified cell cycle regulator [2, 3]. Overexpression of RGC-32 leads to an increase in DNA synthesis and cell cycle progression from the G1/G0 to G2/M phase [3]. Both of these responses can be abolished by transfecting the cells with RGC-32-specific siRNA [4]. RGC-32 forms complexes with cell division cycle protein homolog 2 (CDC2) and enhances CDC2 kinase activity [3]. Thus, RGC-32 appears to be a previously unrecognized regulator of CDC2, a critical kinase involved in cell cycle activation. Despite these findings, little is currently known about the potential role of RGC-32 in autoimmune disorders, including RRMS. In RRMS, myelin and myelin-producing oligodendrocytes in the CNS are targeted for autoimmune attack via antigen-specific $CD4^+$ T cells [5, 6]. Several studies have demonstrated impaired apoptosis of T cells in RRMS patients [6, 7, 8]. Furthermore, relapses may be associated with the persistent presence of myelin-activated T cells resulting from impaired T cell apoptosis. T cell apoptosis in both experimental allergic encephalomyelitis and RRMS is regulated in part by the Fas-FasL system [8], and *ex-vivo* studies have demonstrated an increased resistance of T cells to Fas-mediated apoptosis during RRMS relapses [9]. FasL expression has been found to be low during relapses, consistent with increased resistance of T cells to apoptosis [10]. FasL expression on T cells is regulated by multiple factors, including the CDC2/cyclin B1 complex [11]. SIRT1, a NAD^+-dependent histone deacetylase and key regulator of cell survival and apoptosis, is also regulated in part by the CDC2/cyclin B1 complex. SIRT1 expression has been found to be decreased in the PBMCs of RRMS patients during relapses [12]. Furthermore, SIRT1 expression was reduced by 50% following RGC-32 silencing, suggesting an important role for RGC-32 in SIRT1 expression [12]. Since RGC-32 binds to the CDC2/cyclin B1 complex and up-regulates its activity, it is possible that RGC-32 is involved in regulating T cell survival by modulating the expression of FasL and SIRT1.

Objectives

It has been shown in previous, separate studies that RGC-32, FasL, and SIRT1 mRNA expression is significantly lower within PBMCs of RRMS patients during relapse compared to remission. The present study aims to longitudinally investigate the combined roles of RGC-32, FasL, SIRT1, and CDC2 as possible peripheral blood-based biomarkers of relapse and predictors of response to glatiramer acetate (GA) treatment in a cohort of RRMS patients.

Materials and Methods

Patients and controls. A cohort of 15 patients with RRMS were enrolled in the study. The patients were primarily recruited from the University of Maryland Multiple Sclerosis Center. The criteria for inclusion of MS patients in the study were: (i) age 18 to 65 years; (ii) fulfillment of McDonald criteria for definite MS; (iii) relapsing-remitting course; (iv) having newly diagnosed MS, or MS not treated with currently used immunomodulatory drugs (interferon-β or glatiramer acetate) for 3 months prior to study entry; (v) no exacerbations in the 4 weeks before the study; (vi) no i.v. or p.o. steroids for 4 weeks prior to study enrollment; (vii) no treatment with Tysabri, Gilenya, mitoxantrone, cyclophosphamide, or investigational drugs during the past year; and (viii) a disability score of 0-5.5, as defined by the expanded disability status scale (EDSS). Exclusion criteria for MS patients were: (i) a history of autoimmune disorders, vascular disease, or active acute or chronic infections; (ii) use of antibiotics in the last 30 days; (iii) a history of intracranial or intraspinal tumor or metabolic myelopathy; or (iv) a history of alcohol or drug abuse.

Study design. All MS patients received 20 mg of glatiramer acetate (GA) injected subcutaneously every day for 2 years. During this period of 2 years, patients were clinically evaluated and peripheral blood samples were collected at 0, 3, 6, and 12 months at the time of their outpatient visits. Patients with symptoms suggestive of a clinical relapse called the University of Maryland Multiple Sclerosis Center. Clinical relapse was defined as substantial worsening of pre-existing symptoms or appearance of new neurological deficits in the absence of fever or infections lasting more than 24 h. An EDSS evaluation was completed at each visit. Clinical records, consultation reports, and inpatient records were reviewed by a neurologist (H.R., C.B. or W.R.) to ensure that the data obtained were complete. In the case of patients with relapse, the administration of 1g of Solu-Medrol i.v. for 3 days was used to treat the disease exacerbation. A prednisone taper was also used after i.v. Solu-Medrol in certain cases. In such cases, blood samples were obtained prior to Solu-Medrol treatment. Responders to GA treatment were defined as patients who exhibited 0 or no more than 1 relapse event during the 2 year span following the initiation of GA. Non-responders were defined as patients who exhibited 2 or more relapse events during the 2 year span following the initiation of GA. According to these criteria, the present cohort consisted of 11 responders and 4 non-responders to treatment.

Collection of PBMCs, total RNA purification, and cDNA synthesis. Peripheral blood mononuclear cells (PBMCs) were isolated from each patient's fresh blood sample and RNA isolation was performed the same day. Total RNA (0.5 µg per sample) was purified, denatured, and reverse transcribed using oligo-dT random primers to synthesize cDNA for each sample.

Real-time quantitative PCR. Real-time quantitative PCR was performed using RRMS patient sample cDNA and primer sets specific for each target gene studied (RGC-32, FasL, SIRT1, and CDC2). For each gene, the cycle threshold (Ct) values were determined in the exponential phase of the amplification plot and normalized to the mRNA expression of L13 ribosomal protein, a housekeeping gene. A standard curve was generated using serial dilutions of reference total human cDNA, and the normalized mRNA value (NRV) was calculated according to the following formula for relative expression of target mRNA: NRV = (TarS/L13), where TarS represents the level of mRNA expression of the target gene, and L13 corresponds to that of the amplified L13 mRNA.

Statistical analysis. Comparisons between groups were performed using a two-tailed t-test assuming unequal variances. P values <0.05 were considered significant. Statistical analysis was performed using IBM SPSS Statistics software version 22 and GraphPad Prism software version 6. All values are shown as mean ± SEM and are representative of three experiments unless otherwise noted. Receiver operating characteristic (ROC) curve analysis was used to assess the predictive accuracy of each potential biomarker. The predictive probability of binary outcomes regarding clinical state and response to GA treatment was reported as a C-statistic or Area Under the Curve (AUC, represented as a percentage, with a perfect score being 100% predictability).

References

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Results

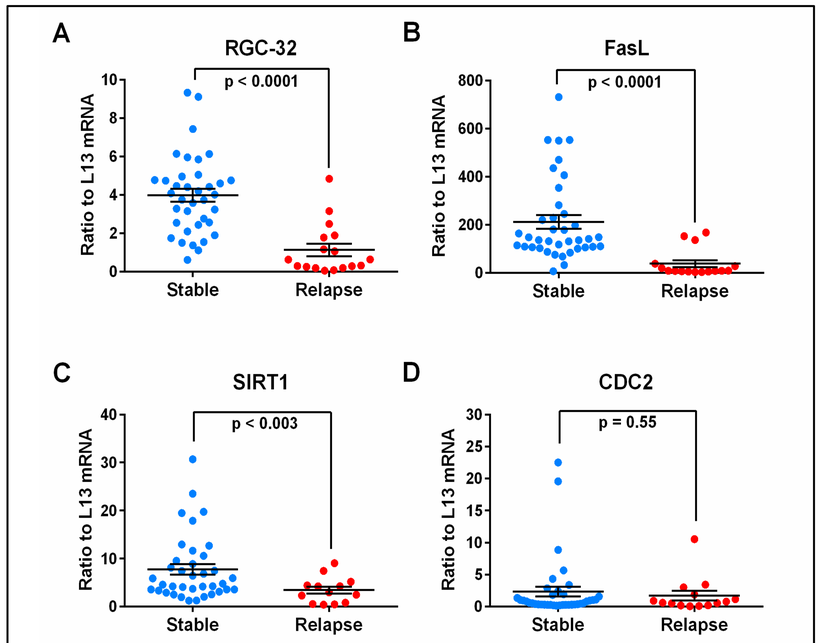

Figure 1. Overall expression of RGC-32, FasL, SIRT1, and CDC2 mRNA in stable and relapsing RRMS patients. Target gene mRNA expression was measured in patients' PBMCs by two-step real-time qRT-PCR and expressed as a ratio to L13 mRNA expression. **A, B, and C.** Overall, significantly lower levels of RGC-32/L13 (p<0.0001), FasL/L13 (p<0.0001), and SIRT1/L13 (p<0.003) were found in patients during relapse compared to remission. **D.** There was no significant change in CDC2/L13 expression between patients during relapse compared to remission (p=0.55).

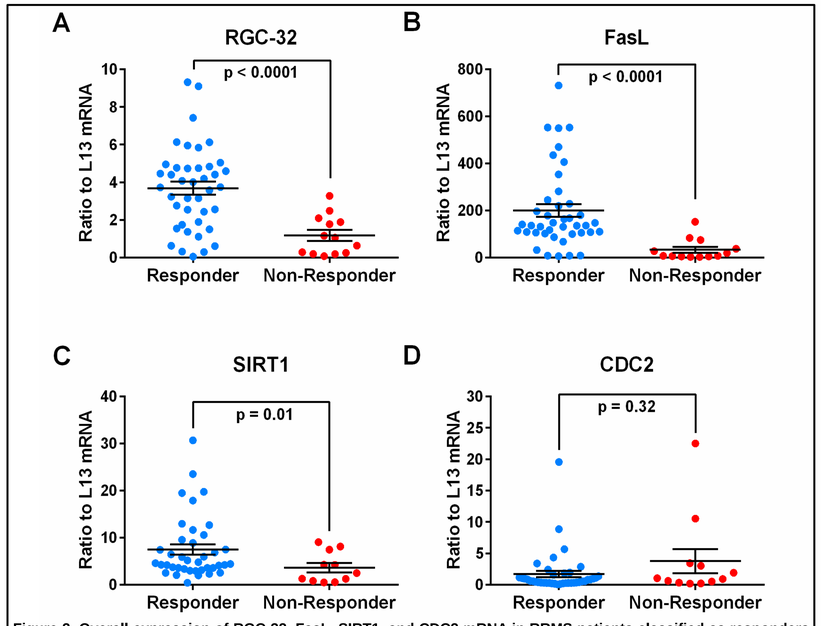

Figure 2. Overall expression of RGC-32, FasL, SIRT1, and CDC2 mRNA in RRMS patients classified as responders and non-responders to GA treatment. Target gene mRNA expression was measured in patients' PBMCs by two-step real-time qRT-PCR and expressed as a ratio to L13 mRNA expression. **A, B, and C.** Overall, significantly higher levels of RGC-32/L13 (p<0.0001), FasL/L13 (p<0.0001), and SIRT1/L13 (p=0.01) were found in responders to GA compared to non-responders. **D.** There was no significant change in CDC2/L13 expression between responders and non-responders (p=0.32).

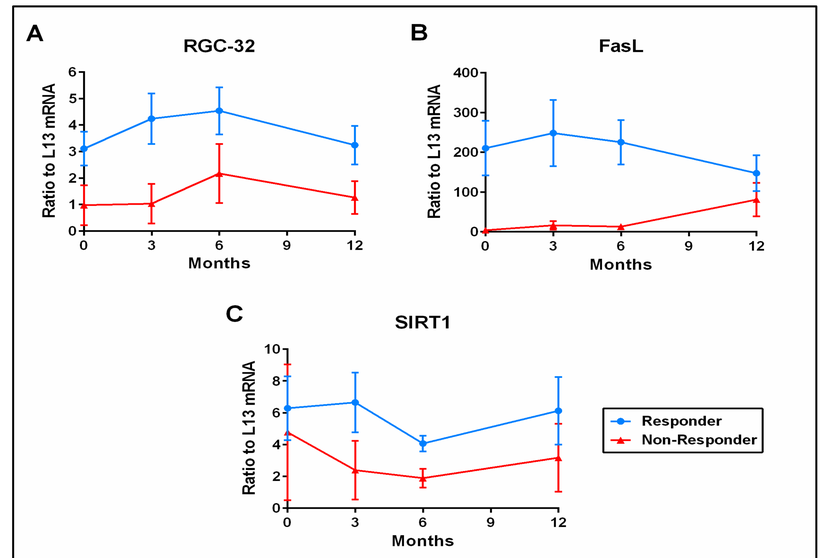

Figure 3. Time course of RGC-32, FasL, and SIRT1 mRNA expression in RRMS patients classified as responders and non-responders to GA treatment. PBMCs from RRMS patients were obtained at baseline (month 0) and subsequently collected at 3, 6, and 12 months following the initiation of GA treatment. Over time, responders to GA showed persistently higher levels of RGC-32 (**A**), FasL (**B**), and SIRT1 (**C**) mRNA expression while non-responders showed persistently lower levels.

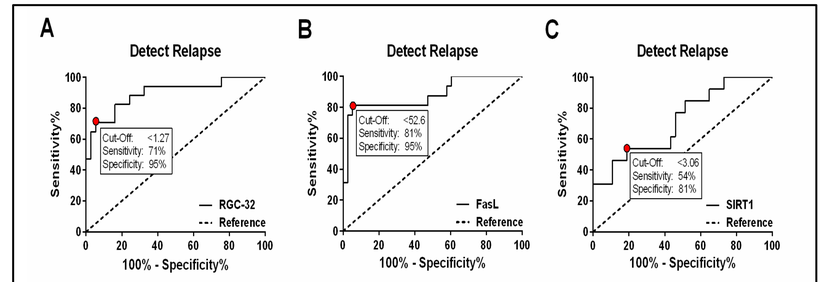

Figure 4. Receiver operating characteristic (ROC) curve analysis to assess the predictive accuracy of RGC-32, FasL, and SIRT1 in detecting RRMS patient relapse. The probability (C-statistic, or AUC) of accurately predicting relapse was 90% using RGC-32 (p<0.0001), 88% using FasL (p<0.0001), and 72% using SIRT1 (p<0.02). **A.** In the present cohort, a RGC-32/L13 ratio < 1.27 detected RRMS patient relapse with a sensitivity of 71% and a specificity of 95%. **B.** A FasL/L13 ratio < 52.6 detected RRMS patient relapse with a sensitivity of 81% and a specificity of 95%. **C.** A SIRT1/L13 ratio < 3.06 detected RRMS patient relapse with a sensitivity of 54% and a specificity of 81%.

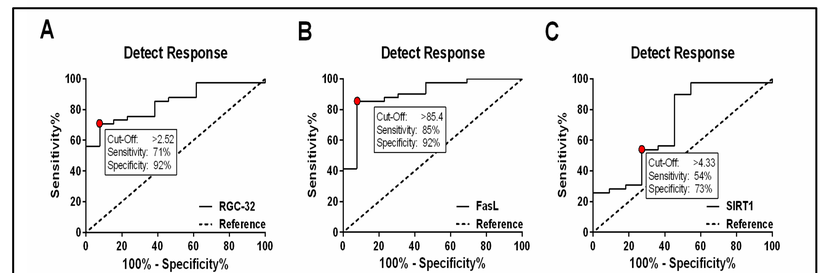

Figure 5. Receiver operating characteristic (ROC) curve analysis to assess the predictive accuracy of RGC-32, FasL, and SIRT1 in detecting RRMS patient response to GA. The probability (C-statistic, or AUC) of accurately predicting response to GA treatment over 2 years was 85% using RGC-32 (p=0.0002), 90% using FasL (p<0.0001), and 70% using SIRT1 (p=0.04). **A.** In the present cohort, a RGC-32/L13 ratio > 2.52 detected RRMS patient response to GA with a sensitivity of 71% and a specificity of 92%. **B.** A FasL/L13 ratio > 85.4 detected RRMS patient response to GA with a sensitivity of 85% and a specificity of 92%. **C.** A SIRT1/L13 ratio > 4.33 detected RRMS patient response to GA with a sensitivity of 54% and a specificity of 73%.

Conclusions

1. RGC-32, FasL, and SIRT1 mRNA expression is significantly decreased in RRMS patients during periods of clinical relapse compared to remission.
2. RGC-32, FasL, and SIRT1 mRNA expression is significantly increased overall in responders to glatiramer acetate treatment compared to non-responders followed over time.
3. ROC analyses suggest that RGC-32, FasL, and SIRT1, but not CDC2, could potentially serve as peripheral blood-based biomarkers to help confirm RRMS patient relapse and identify response to glatiramer acetate therapy.