SIRT1 as a Potential Marker of Disease Activity and Response to Treatment with Glatiramer Acetate in Multiple Sclerosis.

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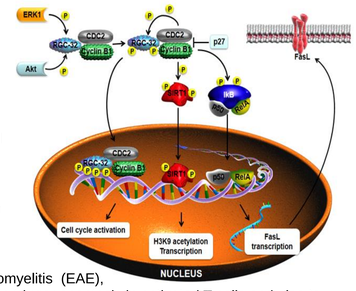
Background

Epigenetic regulators are increasingly being implicated as regulatory components of expression of neuronal and immune specific genes. Histone protein post-translational modifications have the ability to affect chromatin structure and regulate gene expression. SIRT1 (Sirtuin 1) is a member of the HDAC class III family of proteins. It is a NAD-dependent histone and protein deacetylase that catalyzes the removal of acetyl groups from a variety of protein substrates, including histones H1, H3, and H4. In addition, SIRT1 has been found to promote histone H3 lys9 (H3K9) methylation, resulting in epigenetic gene silencing. SIRT1 is involved in the regulation of a number of cellular processes, including transcription, metabolism, DNA repair, and aging. SIRT1 can induce chromatin silencing through the deacetylation of histones and can modulate cell survival by regulating the transcriptional activities of p53, NF-κB, FOXO proteins, and p300. Recently, resveratrol, a SIRT1 activator, was shown to ameliorate the disease course in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Studies have shown the ability of resveratrol to trigger apoptosis in activated T cells and also to induce a decrease in spinal cord inflammation during EAE. The mechanism for its immunomodulatory and neuroprotective effects involves the activation of SIRT1. SIRT1 expression has also been found to be decreased in the PBMCs of RRMS patients during relapses. Furthermore, SIRT1 expression was reduced by 50% following RGC-32 silencing, suggesting an important role for RGC-32 in SIRT1 expression. Little is known about the changes that occur in SIRT1 expression or in the acetylation and methylation of histones in the T cells from MS patients. However, it is well documented that autoreactive memory T cells play an important role in MS pathogenesis.



Patients with Relapsing-Remitting Multiple Sclerosis (RRMS) are commonly managed with first-line drug treatments glatiramer acetate (GA) or interferon beta to reduce the annualized relapse rate by 29%. However, due to the heterogeneous nature of RRMS it is difficult to predict patient response to treatment. Currently there is a critical need for the development of reliable biomarkers to aid clinicians in their management of RRMS patients. In this study, we have investigated the role of SIRT1 as a possible biomarker of relapses and as a predictor of response to GA treatment in RRMS patients.

Objectives

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

Materials and Methods

Patients. 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 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 at the time of their outpatient visits using BD Vacutainer CPT tubes (Becton Dickinson) and RNA isolation was performed the same day. Total RNA (0.5 µg per sample) was purified (RNeasy, Qiagen), 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 SIRT1. 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.

Western Blot. Western Blot protein analysis was performed using patient PBMC samples that were lysed in RIPA buffer and processed. Whole-cell lysates (total protein = 10-30 µg) were analyzed by 12% SDS-PAGE, followed by western blotting. Each membrane was analyzed for SIRT1 (Active Motif), H3K9me2 (CST), and H3K9ac (Active Motif) using specific antibodies. β-actin (Rockland) was also analyzed as a loading control for normalization. Anti-rabbit or anti-mouse HRP-conjugated antibody (Santa Cruz) were used as the secondary antibodies, and signals were visualized by enhanced chemiluminescence (Pierce) and autoradiography. Blots were then stripped and re-probed. The radiographic band density was measured using UN-SCAN-IT software (Silk Scientific, Orem, UT) and results were expressed as a ratio to β-actin

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).

Acknowledgements

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Results

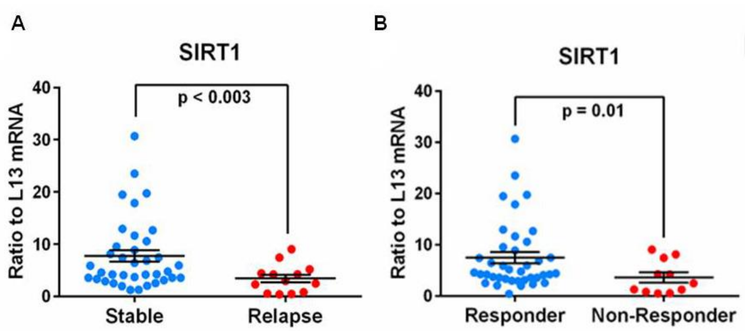

Figure 1. SIRT1 mRNA expression in stable and relapsing RRMS patients and RRMS patients classified as responders and non-responders to GA treatment. PBMCs from RRMS patients were obtained at baseline (month 0) and subsequently collected at 3, 6, and 12 months following the initiation of GA treatment. 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. Overall, significantly lower levels of SIRT1/L13 (p<0.003) were found in patients during relapse compared to remission.

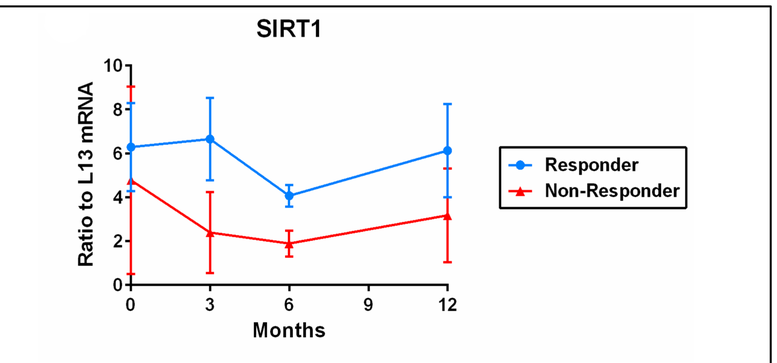

Figure 2. Time course of SIRT1 mRNA expression in RRMS patients classified as responders and non-responders to GA treatment. PBMCs from RRMS patients were obtained at baseline (month 0) and subsequently collected at 3, 6, and 12 months following the initiation of GA treatment. Over time, responders to GA showed persistently higher levels of SIRT1 mRNA expression while non-responders showed persistently lower levels.

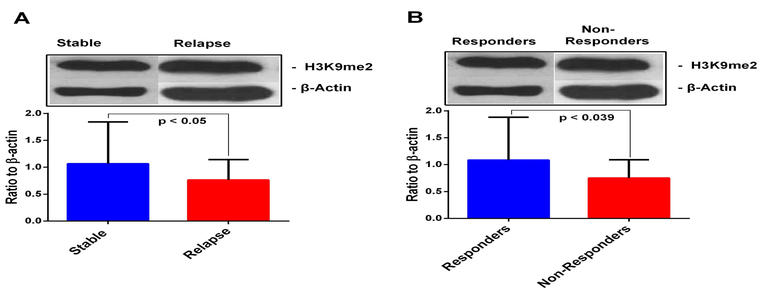

Figure 3. Overall expression of H3K9me2 expression in stable and relapsing RRMS patients and RRMS patients classified as responders and non-responders to GA treatment. An example of the western blot relevant to the expression of H3K9me2 is on top of each graph. PBMCs from RRMS patients were obtained at baseline (month 0) and subsequently collected at 3, 6, and 12 months following the initiation of GA treatment. Methylation of H3K9 was measured in patients' PBMCs by western blot and expressed as a ratio to β-Actin protein expression. Overall, significantly lower levels of H3K9me2 were found in patients during relapse (p<0.05) compared to remission and in patients who were responders to GA treatment (p<0.039) .

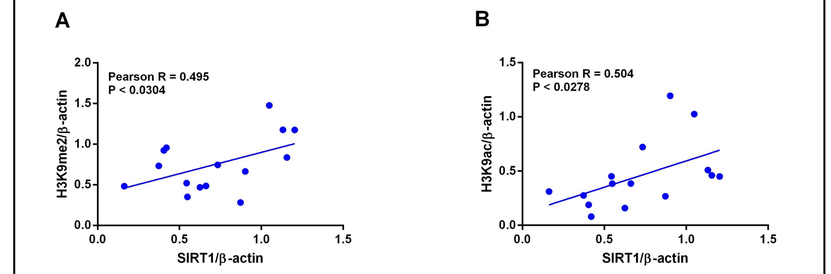

Figure 4. Correlation of SIRT1 protein with histone H3K9me2 and H3K9ac (expressed as ratios to β-Actin) in non-responders. Correlation of SIRT1 with H3K9me2 and H3K9ac expression by Pearson Correlation coefficient. SIRT1 protein levels were positively correlated with levels of H3K9ac (A; R=0.504, p<0.0304) and H3K9me2 (B; R=0.495, p<0.0278)

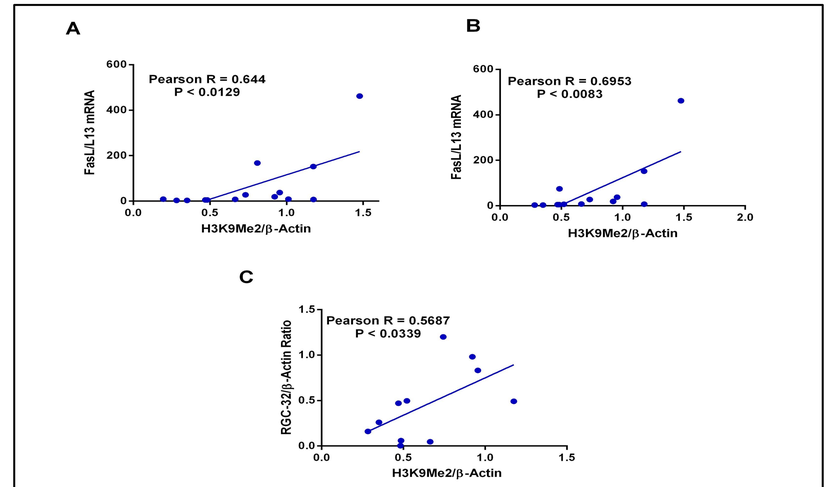

Figure 5. Correlation of H3K9me2 with FasL mRNA and RGC-32 protein expression.
(A) H3K9me2 expression was positively correlated with levels of FasL mRNA (R=0.644, p<0.0129) in relapse patients.
(B) H3K9me2 expression was positively correlated with levels of FasL mRNA (R=0.6953, p<0.0083) in patients who were non-responders.
(C) H3K9me2 expression was positively correlated with levels of RGC-32 protein (R=0.5687, p<0.0339) in patients who were non-responders.

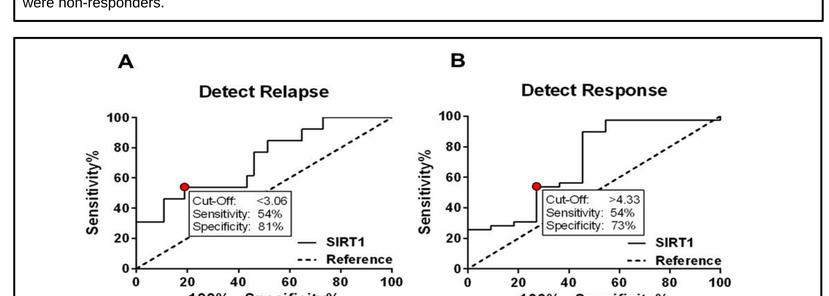

Figure 6. Receiver operating characteristic (ROC) curve analysis was used to assess the predictive accuracy of SIRT1 in detecting patient relapse and response to GA.
(A) The probability (C-statistic, or AUC) of accurately predicting a relapse was 72% using SIRT1 (p<0.02). A SIRT1/L13 ratio < 3.06 detected patient relapse with a sensitivity of 54% and a specificity of 81%. **(B)** The probability (C-statistic, or AUC) of accurately predicting response to GA treatment over 2 years was 70% using SIRT1 (p=0.04). A SIRT1/L13 ratio > 4.33 detected patient response to GA with a sensitivity of 54% and a specificity of 73%.

Conclusions

1. SIRT1 mRNA expression is significantly decreased in RRMS patients during periods of clinical relapse compared to remission.
2. SIRT1 mRNA expression is significantly increased overall in responders to GA treatment compared to non-responders over time.
3. H3K9me2 and H3K9ac expression is correlated to SIRT1 protein level during relapses and in non-responders to GA treatment.
4. H3K9me2 expression is significantly increased during relapses and in responders to GA treatment. In addition, H3K9me2 is correlated to FasL mRNA and RGC-32 protein in non-responders to GA.
5. ROC analyses suggest that SIRT1 mRNA could potentially serve as peripheral blood-based biomarker to help predict relapse and response to GA therapy.