Phosphorylated SIRT1 as a biomarker of relapse and response to treatment with glatiramer acetate in multiple sclerosis

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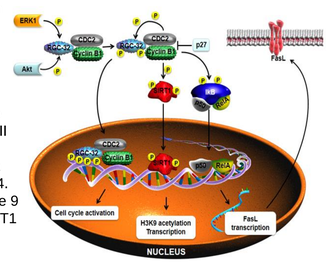
Background

Multiple sclerosis (MS) is a demyelinating disease characterized by chronic inflammation of the central nervous system in which many factors (genetic and environmental) act in concert to influence disease susceptibility and progression. 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 an 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 lysine 9 (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 to decrease spinal cord inflammation during EAE. The mechanism for resveratrol immunomodulatory and neuroprotective effects is thought to involve the activation of SIRT1. SIRT1 can be activated by phosphorylation at 13 residues. This post-translational modification appears to play an important role in the function of SIRT1. Furthermore, SIRT1 mRNA expression has been found to be decreased in the PBMCs of RRMS patients during relapses. It is well documented that autoreactive memory T cells play an important role in MS pathogenesis. However, little is known about the changes that occur in SIRT1 phosphorylation or in histone methylation in T cells from MS patients.



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. 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 their management of RRMS patients. In this study, we have investigated the role of phosphorylated SIRT1 (p-SIRT1) and trimethylated H3K9 (H3K9me3) as a possible biomarkers of relapses and as a predictors of response to GA treatment in RRMS patients.

Objectives

It has been shown in previous studies that SIRT1 mRNA expression is significantly lower within PBMCs of RRMS patients during relapse compared to remission. The present study aims to longitudinally investigate the role of p-SIRT1 as a possible peripheral blood-based biomarkers of relapse and predictors of response to GA treatment in a cohort of RRMS patients. We also investigated the role of H3K9me3 as a second biomarker of relapse and predictor of response to GA treatment in 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. 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). Samples were stored at -80°C until sample lysis.

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 = 40-50 µg) were analyzed by 4-20% SDS-PAGE (BioRad), followed by western blotting. Each membrane was analyzed for p-SIRT1 (phospho-Ser47; CST), SIRT1 (Active Motif), and H3K9me3 (CST) 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 (Denville Scientific) 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 GraphPad Prism software version 6. All values are shown as mean ± SEM and are representative of at least 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

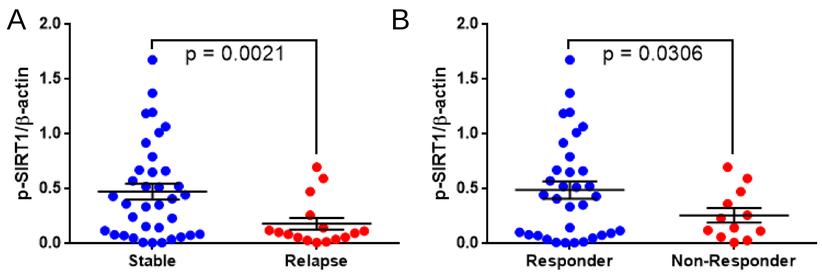

Figure 1. p-SIRT1 protein levels in stable and relapsing RRMS patients (A) and RRMS patients classified as responders and non-responders to GA treatment (B). PBMCs from RRMS patients were obtained at baseline (month 0) and subsequently collected at 3, 6, and 12 months following the initiation of GA treatment. Protein levels of p-SIRT1 were measured in patients' PBMCs by western blot and expressed as a ratio to β-Actin. Significantly lower levels of p-SIRT1/β-actin (**A,** p=0.0021) were found in patients during relapse compared to remission. Significantly higher levels of p-SIRT1(**B**, p=0.0306) were found in patients classified as responders to GA treatment compared to non-responders.

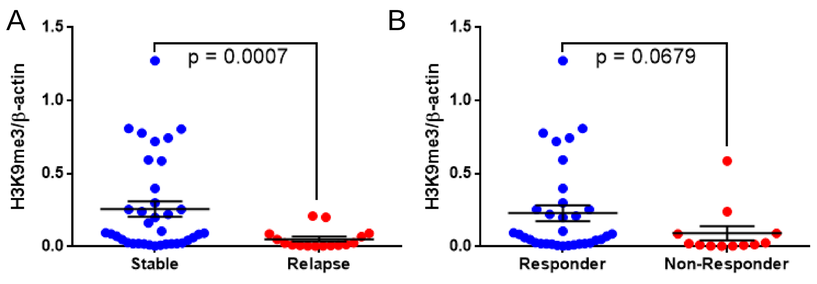

Figure 2. H3K9me3 protein levels in stable and relapsing RRMS patients (A) and RRMS patients classified as responders and non-responders to GA treatment (B). PBMCs from RRMS patients were obtained at baseline (month 0) and subsequently collected at 3, 6, and 12 months following the initiation of GA treatment. Protein levels of H3K9me3 were measured in patients' PBMCs by western blot and expressed as a ratio to β-Actin. Significantly lower levels of H3K9me3/β-actin (**A,** p=0.0007) were found in patients during relapse compared to remission. No significant difference between levels of H3K9me3/β-actin (**B**, p=0.0679) were found between patients classified as responders and non-responders to GA treatment.

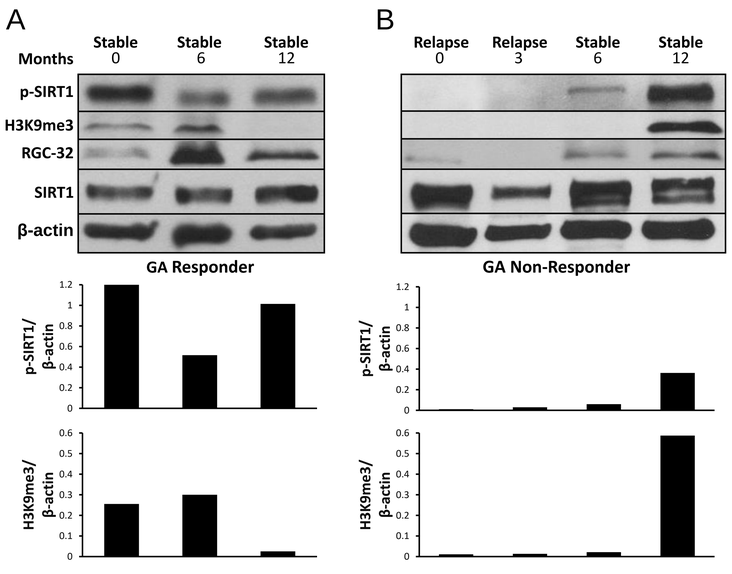

Figure 3. Western blot images from two selected patients treated with GA. Examples of western blots probing for p-SIRT1, H3K9me3, and β-actin on PBMC protein lysate from patients with RRMS. The patient shown in **A** is a responder to treatment and the patient shown in **B** is a non-responder to GA treatment. p-SIRT1 and H3K9me3 protein levels ratio to β-actin as loading control are show below the respective western blot images.

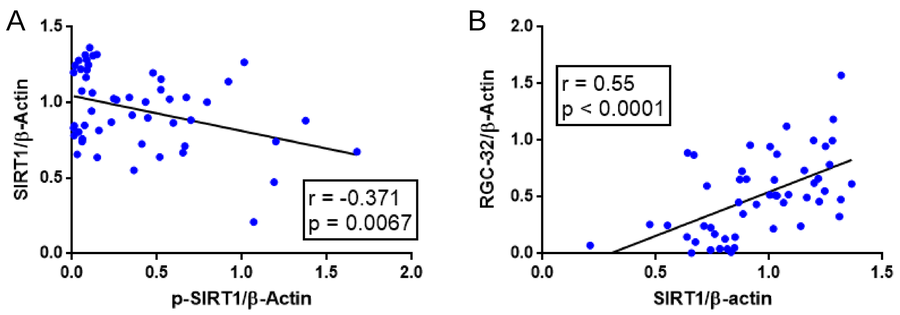

Figure 4. Correlation between phosphorylated SIRT1 protein levels with unphosphorylated SIRT1 protein levels (A) and between unphosphorylated SIRT1 protein levels and RGC-32 protein levels. Correlations were performed using Pearson correlation. Phosphorylated SIRT1 protein levels were negatively correlated with levels of unphosphorylated SIRT1 (r=-0.0371, p=0.0067). Unphosphorylated SIRT1 protein levels were positively correlated with levels of RGC-32 (r=0.55, p<0.0001).

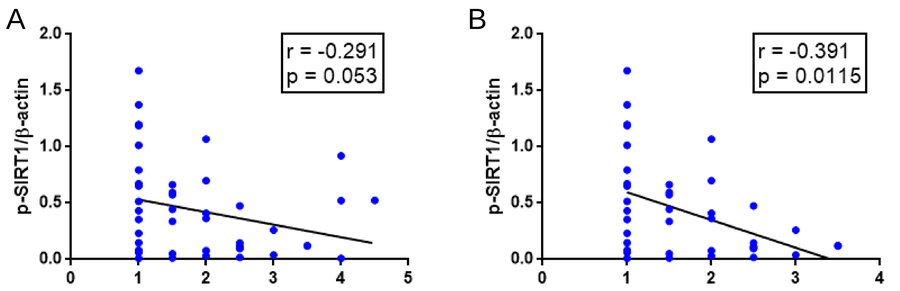

Figure 5. Correlation between EDSS and p-SIRT1. There is a trend that higher EDSS correlates with lower p-SIRT/ β-actin levels (**A,** p = 0.053). When patients with EDSS >3.5, representing patients with significant disability and impairment to waking, were excluded, there is a significant relationship between EDSS and p-SIRT1/ β-actin (**B**, p = 0.0115).

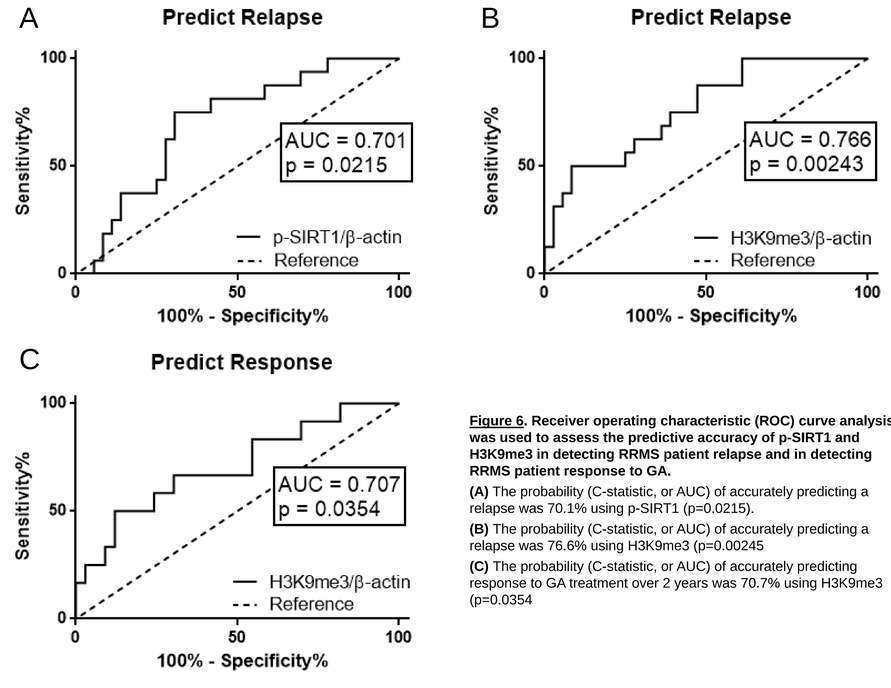

Figure 6. Receiver operating characteristic (ROC) curve analysis was used to assess the predictive accuracy of p-SIRT1 and H3K9me3 in detecting RRMS patient relapse and in detecting RRMS patient response to GA.

(A) The probability (C-statistic, or AUC) of accurately predicting a relapse was 70.1% using p-SIRT1 (p=0.0215).

(B) The probability (C-statistic, or AUC) of accurately predicting a relapse was 76.6% using H3K9me3 (p=0.00245

(C) The probability (C-statistic, or AUC) of accurately predicting response to GA treatment over 2 years was 70.7% using H3K9me3 (p=0.0354

Conclusions

1. **p-SIRT1 protein levels are significantly decreased in RRMS patients during periods of clinical relapse and significantly increased in patients who are responders to GA treatment.**
2. **H3K9me3 protein levels are significantly decreased in RRMS patients during periods of clinical relapse.**
3. **p-SIRT1 protein inversely correlated with unphosphorylated SIRT1 protein levels.**
4. **p-SIRT1 could potentially serve as peripheral blood-based biomarker to help confirm RRMS patient relapse.**
5. **H3K9me3 could potentially serve as a peripheral biomarker to help confirm RRMS patient relapse and predict response to GA therapy.**