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Serum CCL20 and its association with SIRT1 activity in multiple sclerosis patients

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Abstract:
CCL20 is a potentially important component in the pathogenesis of multiple sclerosis (MS). SIRT1 exhibits a negative regulatory effect on a variety of inflammatory cytokines and can relieve experimental autoimmune encephalomyelitis. The association between the level of CCL20 and SIRT1 activity in MS patients has not been investigated. In the present study, blood samples were collected from 38 RRMS patients and 40 healthy controls. The serum CCL20 levels were measured by ELISA, SIRT1 activity was evaluated by fluorometric assay. We revealed elevated serum CCL20 concentrations in MS, and discovered an inverse correlation between CCL20 and SIRT1 activity in MS patients.

Keywords: multiple sclerosis, CCL20, SIRT1

1. Introduction
Multiple sclerosis (MS) is a demyelinating disease characterized by chronic inflammation of the central nervous system. It has been reported that lymphocytes such as Th17 cells play key roles in the pathogenesis of MS (Jadidi-Niaragh et al, 2011; Rostami et al, 2013). The infiltration of lymphocytes into the CNS is an essential step in the neuropathogenesis of MS and is controlled by chemokines (Holman et al, 2011), a group of small polypeptides that attract various leukocytes to sites of inflammation (Raman et al, 2011). CCL20 is a highly regulated inflammatory
chemokine that, via its receptor CCR6, drives the recruitment of lymphocytes, especially Th17 cells, to sites of CNS injury (Ambrosini et al., 2003; Liston et al., 2009; Reboldi et al., 2009; Yamazaki et al., 2008). Limited evidence has shown increased serum CCL20 in MS patients (Jafarzadeh et al., 2014). However, little is known about the expression of CCL20 in relation to the clinical characteristics of MS patients.

There is increasing evidence that epigenetic mechanisms of gene expression are involved in the regulation of proinflammatory cytokines in multiple sclerosis (Huynh et al., 2013; Kurtuncu et al., 2008). Epigenetic control refers to several biochemical reactions in DNA that do not change its sequence, including histone acetylation, methylation, citrullination, sumoylation, and ubiquitination (Huynh et al., 2013). Histone acetylation is catalyzed by histone acetyl transferase (HAT) while deacetylation is influenced by histone deacetylase (HDAC). SIRT1, a member of the HDAC class III family of proteins (Smith et al., 2000), is an NAD+-dependent histone and protein deacetylase (Penberthy et al., 2009; Smith et al., 2000) that catalyzes the removal of acetyl groups from a variety of protein substrates (Turner et al., 1998), including histones H1, H3, and H4 (Turner et al., 1998; Wang et al., 2011; Zhang et al., 2010). SIRT1 induces chromatin silencing through the deacetylation of histones (Baur et al., 2010) and modulates cell survival by regulating the transcriptional activities of p53 (Luo et al., 2000), NF-κB (Yeung et al., 2004), and FOXO proteins (Brunet et al., 2004; Motta et al., 2004). SIRT1 exhibited negative regulatory effects on a variety of inflammatory factors, such as TNF-α and IL-8 (Lee et al., 2011; Shen et al., 2009). A recent study demonstrated that overexpression of SIRT1 had immunomodulatory effects by reducing the numbers of IL-17-positive T cells in the white matter of spinal cord in the experimental autoimmune encephalomyelitis (EAE) model (Nimmagadda et al., 2013). CCL20 is the major chemokine that attracts Th17 cells into the CNS; therefore, we hypothesized that SIRT1 reduces the number of central Th17 cells in EAE by inhibiting the expression of CCL20. The association between the levels of CCL20 and SIRT1 activity in MS patients has not been investigated.

Therefore, we performed a hospitalized-based study to investigate the peripheral levels of CCL20 and SIRT1 activity, the correlation between CCL20 levels and clinical characteristics, and the association between CCL20 and SIRT1 activity in MS patients.

2. Materials and Methods

2.1 Subjects

Blood samples were collected from 38 relapsing-remitting MS (RRMS) patients in the Third Affiliated Hospital of Sun Yat-sen University and 40 healthy controls. Expert neurologists confirmed MS according to clinical and paraclinical findings based on McDonald’s criteria (Polman et al., 2011). MS relapse was defined as the last attack within one month previous. Healthy controls were recruited from blood donors. All controls were healthy with no acute or chronic illnesses. This research was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-sen University (No. 2007-33) and the procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008. All participants involved in this study
provided written informed consent. A peripheral blood sample (6 ml) was collected in sodium heparin tubes from all participants. Peripheral blood mononuclear cells (PBMCs) were purified using Ficoll-Hypaque gradient centrifugation (Tianjin Hao Yang Biological Manufacture, Tianjin, China). PBMCs were adjusted to a final concentration of 10^6/ml in RPMI 1640 medium supplemented with 10% heated inactivated fetal calf serum. Collected PBMCs was stored at −70°C until use. The sera were separated and stored at −70°C until analysis.

2.2 Detection of CCL20 and SIRT1 activity

The serum levels of CCL20 (R&D Systems, Abingdon, UK) were measured by commercial ELISA kits according to the manufacturer's guidelines. Total proteins were extracted using lysis buffer containing 10 mM Tris-HCl buffer (pH 7.5) and protease inhibitors. SIRT1 activity was evaluated from total protein of PBMC samples using a fluorometric assay (SIRT1 fluorimetric kit, BML-AK-555, Enzo Life Sciences, Villeurbanne, France).

2.3 Measurement of global histone H3/H4 acetylation and H3K9 acetylation

PBMC total histone was extracted using an EpiQuik™ total histone extraction kit (Epigentek Group Inc., NY, USA) according to the manufacturer’s protocol. PBMC Global histone H3/H4 acetylation and H3K9 acetylation status were assessed with the EpiQuik™ global histoneH3/H4 acetylation and H3K9 acetylation quantification Kit (Epigentek Group Inc., NY, USA), respectively, according to the manufacturer’s protocols.

2.4 Clinical data collection

MS patients’ medical records were reviewed retrospectively, and the following data were retrieved: gender, age at onset, disease duration, number of relapses, EDSS (Kurtzke et al, 1983) at last visit, clinical manifestations, laboratory and MRI findings, and therapy including the use of methylprednisolone, interferon-β (INF-β), and/or immunosuppressants.

2.5 Statistical Analysis

Comparisons of CCL20, SIRT1 activity, global histone H3/H4 acetylation and H3K9 acetylation between MS with relapse, MS in remission, and the control groups were analyzed by one-way ANOVA test. An independent t-test was performed using the mean difference of CCL20 levels among subgroups of age, gender, annual relapse rate (ARR), expanded disability status scale (EDSS), disease duration, and treatment. The association between two variables was estimated with Pearson’s correlation coefficient. Further multiple stepwise regression analysis was performed to detect independent factors contributing to the increase in CCL20 (independent variables tested were age of onset, gender, ARR, EDSS, disease duration, treatment, and SIRT1 activity). P values less than 0.05 were considered significant. The data were analyzed by statistical software (SPSS version 15, Chicago, IL, USA).

3. Results
3.1 Clinical data

Patient demographic and clinical features are presented in Table 1. In MS patients, the female to male ratio was 20:18, the disease age of onset was 26.05±10.28 years, the mean duration of disease was 42.68 (range 0.3–240) months, ARR was 0.93±0.82, and mean EDSS score was 3.0±1.78. Methylprednisolone pulse therapy was administered to all MS patients in the acute phase. During remission, 24 patients received additional disease-modifying drugs and/or immunosuppressants (Table 1).

3.2 Correlation of serum CCL20 levels and clinical characteristics of MS patients

The serum level of CCL20 was significantly higher in MS patients compared with controls (MS in acute phase vs. control: 67.80±22.21 vs. 47.35±18.55, p = 0.004; MS in remission vs. control: 57.98±23.27 vs. 47.35±18.55, p = 0.046). The concentration of CCL20 was slightly higher in MS patients in the acute phase compared with MS patients in remission, but this trend was not statistically significant (p = 0.179) (Fig. 1). The clinical indicators examined (age, gender, ARR, EDSS, disease duration, and treatment) did not affect the concentration of serum CCL20 (Table 2).

3.3 Correlation of SIRT1 activity and CCL20 in MS patients

A statistically significant decrease in SIRT1 activity was seen in MS patients with relapse compared with MS patients in remission (7.69±2.39 VS. 11.27±4.23, p = 0.009) and controls (7.69±2.39 VS. 11.85±3.83, p = 0.001). MS patients in remission had similar levels of SIRT1 activity compared with controls (Fig. 2A). Pearson’s correlation test showed that serum CCL20 levels were negatively correlated with SIRT1 concentrations (R = −0.331, p = 0.042) (Fig. 2B). Further multiple stepwise regression analysis showed that SIRT1 was an independent factor contributing to the increase in CCL20 (R²=0.110, p = 0.042). We did not find a correlation of CCL20 and SIRT1 activity in the control group (R=0.045, p=0.782).

3.4 Correlation of acetylated H3, H4 and H3K9 levels with SIRT1 activity in MS patients

As mentioned above, SIRT1 is a member of the HDAC. To screen candidate deacetylation sites for SIRT1 and their possible effects on CCL20 expression in MS patients, we evaluated acetylated H3/H4 and H3K9 levels and their correlation with SIRT1 activity and CCL20 in MS patients. H3, H4 and H3K9 acetylation were not significantly changed in MS patients compared with controls (Fig. 3A-C). SIRT1 activity as negatively correlated with H3K9 acetylation (R = −0.335, p = 0.040) (Fig. 3D). There was no correlation between SIRT1 activity and acetylated H3/H4. Additionally, we did not observe a correlation between CCL20 and H3, H4 and H3K9 acetylation.

4. Discussion

The major role of Th17 in the pathogenesis of EAE has been demonstrated (Rodgers et al, 2012). CCL20 (as a Th17 chemokine) may play an role in the pathogenesis of MS disease. Our results
confirmed higher levels of CCL20 in MS patients compared with the healthy group, similar to that reported in previous studies (Jafarzadeh et al, 2014; Kalinowska-Lyszczarz et al, 2011). CCL20 is produced by a variety of cells including endothelial cells, monocytes, neutrophils, NK cells, T cells, dendritic cells, Langerhans cells and macrophages in response to stimulators such as IL-1α, IL-β, IL-6, IL-17, IL-21, IFN-γ and TNF-α (Lee et al, 2013; Schutyser et al, 2003; Tesmer et al, 2008), which are elevated in MS patients (Kallaur et al, 2013; Wang et al, 2013); thus, higher serum level of CCL20 in MS patients may originate from monocytes and T lymphocytes among PBMCs following these inflammatory cytokines in present study.

Unlike previous studies, in which CCL20 serum levels were significantly higher in patients during the stable phase of their disease versus the relapsed phase (Jafarzadeh et al, 2014; Kalinowska-Lyszczarz et al, 2011), we found that MS patients in the acute phase had higher CCL20 level compared with patients in remission. Interestingly, a study by Furlan et al. (Furlan et al, 2005) reported higher PBMC mRNA levels of CCL20 in MS patients with relapse compared with MS patients in remission, which is in agreement with our results. These findings indicate that CCL20 may be associated with MS disease activity. The discrepancy with the other studies mentioned above may partially because of the different time interval between the attack onset and blood sampling (less than one month in our study, no detailed description in previous studies). We have added to the results of previous studies by showing that treatment with DMTs and/or immunosuppressants including interferon beta, methotrexate(MTX), azathioprine(AZA), Mitoxantrone, mycophenolate mofetil(MMF), or Tacrolimus did not reduce CCL20 levels in MS patients. It indicated that current treatments for MS may have no action on the target of CCL20. Therefore, more studies are encouraged for developing novel treatment.

Another important finding in the present study was the negative correlation between circulating CCL20 levels and SIRT1 activity in MS patients. We did not find a correlation of CCL20 and SIRT1 activity in the control group, which indicates that the negative correlation of CCL20 and SIRT1 activity exist under pathological conditions of MS specifically. Which factors regulate CCL20 expression in MS patients is still unclear. We assumed that SIRT1 may be a potential regulator of CCL20 by inhibiting CCL20 gene transcription via deacetylating histone H3K9 in the CCL20 promoter region, SIRT1 deacetylate histones causing chromatin silencing and inhibition of target gene transcription (Baur et al, 2010). We demonstrated that SIRT1 activity was significantly decreased in the PBMCs of MS patients in the acute phase compared to healthy controls, and that the serum CCL20 level was negatively correlated with the level of SIRT1 activity in MS patients. Moreover, we found a negative correlation between SIRT1 activity and H3K9 acetylation in patients with MS, supporting our assumption.

However, we did not observe a correlation between CCL20 and H3K9 acetylation. Therefore, other mechanisms might be involved in the regulatory effect of CCL20 expression by SIRT1. NF-κB was previously reported to be involved in the expression of the CCL20 gene (Li et al, 2014; Meares et al, 2012). SIRT1 suppresses the transcriptional activity of NF-κB to reduce proinflammatory cytokines downstream of the NF-κB pathway, by deacetylating acetylated lysines in p65, a NF-κB subunit (Huang et al, 2012; Lei et al, 2012). Thereby, SIRT1 may downregulate the transcriptional activity of NF-κB though the deacetylation of p65 in the
promoter region of the CCL20 gene, thereby reducing the expression of CCL20. Further studies are needed to prove that SIRT1 ameliorates EAE through these pathways.

Although our findings suggest a possible association between CCL20 and SIRT1 activity, this study could not make a conclusion that there is a causal relationship between CCL20 and SIRT1 activity without more direct evidence of this association. Further work is warranted to explore the effect of SIRT1 activation on CCL20 production from PBMC.

Conclusion
Our study confirmed elevated serum CCL20 concentration in MS. In addition, we discovered a correlation between CCL20 and SIRT1 activity in MS patients. Thus, SIRT1 may be a potential regulator of CCL20. Further investigation into the potential regulatory effect of SIRT1 on CCL20 expression in MS may provide insights regarding the pathogenesis of and novel drug targets for the treatment of MS.

Acknowledgment and fundings
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References:


Zhang, T., Kraus, W.L., 2010. SIRT1-dependent regulation of chromatin and transcription: linking NAD(+) metabolism and signaling to the control of cellular functions. Biochim Biophys Acta 1804(8), 1666-1675.
Figure 1. Serum level of CCL20 in MS patients and controls.
CTLs: controls; MS: multiple sclerosis
Figure 2. SIRT1 activity and its correlation with CCL20 in MS patients. A) SIRT1 activity in MS patients and controls. B) Correlation of SIRT1 activity and CCL20 in MS patients. CTLs: controls; MS: multiple sclerosis.
Figure 3. Acetylated H3, H4 and H3K9 levels and the correlation of acetylated H3K9 levels with SIRT1 activity in MS patients. A) H3, B) H4, and C) H3K9 acetylation in MS patients and controls. D: Correlation of H3K9 acetylation and SIRT1 activity in MS patients. CTLs: controls; MS: multiple sclerosis.
<table>
<thead>
<tr>
<th>Table 1 Demographic and clinical features of MS patients and controls</th>
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<tr>
<td><strong>MS</strong></td>
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<tr>
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<tr>
<td>Gender (F/M)</td>
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<tr>
<td>Age</td>
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<tr>
<td>Age of onset</td>
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<tr>
<td>Disease duration</td>
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<tr>
<td>ARR</td>
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<td>EDSS</td>
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<td>Treatment (disease-modifying drug and immunosuppressant)</td>
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</tbody>
</table>

MS: multiple sclerosis; CTLs: controls; F: female; M: male; ARR: annual relapse rate; EDSS: expanded disability status scale; MTX: methotrexate; AZA: azathioprine; MMF: mycophenolate mofetil.
<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean ± SD (pg/ml)</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Age of onset</strong></td>
<td></td>
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<tr>
<td>&lt;30 years, n=28</td>
<td>59.11 ± 24.01</td>
<td></td>
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<tr>
<td>≥30 years, n=10</td>
<td>66.58 ± 20.49</td>
<td>0.388</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
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<tr>
<td>Male, n=18</td>
<td>67.29 ± 19.59</td>
<td></td>
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<tr>
<td>Female, n=20</td>
<td>55.49 ± 25.05</td>
<td>0.118</td>
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<tr>
<td><strong>ARR</strong></td>
<td></td>
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<tr>
<td>&lt;1, n=23</td>
<td>62.98 ± 23.00</td>
<td></td>
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<tr>
<td>≥1, n=15</td>
<td>58.16 ± 23.76</td>
<td>0.537</td>
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<tr>
<td><strong>EDSS</strong></td>
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<tr>
<td>&lt;3, n=23</td>
<td>62.08 ± 22.80</td>
<td></td>
</tr>
<tr>
<td>≥3, n=15</td>
<td>59.55 ± 24.30</td>
<td>0.747</td>
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<tr>
<td><strong>Disease duration</strong></td>
<td></td>
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<tr>
<td>&lt;3 years, n=22</td>
<td>58.17 ± 21.79</td>
<td></td>
</tr>
<tr>
<td>≥3 years, n=16</td>
<td>65.96 ± 24.96</td>
<td>0.370</td>
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<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>with DMTs, n=24</td>
<td>60.92 ± 19.88</td>
<td></td>
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<tr>
<td>without DMTs, n=14</td>
<td>61.18 ± 25.21</td>
<td>0.974</td>
</tr>
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ARR: annual relapse rate; EDSS: expanded disability status scale; DMTs: disease modifying therapies; SD: standard deviation.
Figure 1. Serum level of CCL20 in MS patients and controls. CTLs: controls; MS: multiple sclerosis

Figure 2. SIRT1 activity and its correlation with CCL20 in MS patients. A) SIRT1 activity in MS patients and controls. B) Correlation of SIRT1 activity and CCL20 in MS patients. CTLs: controls; MS: multiple sclerosis

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Highlights

1. CCL20 is a potentially important component in the pathogenesis of MS.
2. We revealed elevated serum CCL20 concentrations in MS patients.
3. We discovered an association between CCL20 and SIRT1 activity in MS patients.