CHAPTER 7
SERUM SECRETOGANIN III AS A MARKER FOR CLINICAL SUBTYPING IN MULTIPLE SCLEROSIS

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ABSTRACT

BACKGROUND & OBJECTIVE: No serum biomarkers for clinical subtyping or disease progression in MS are yet available. Such biomarkers are strongly needed in a chronic disease like MS. In recently performed cerebrospinal fluid (CSF) and serum high-throughput MALDI-TOF-based mass spectrometry studies, we identified novel biomarkers in MS, among which a fragment of secretogranin III (SGIII). The aim of the current study was to validate this novel marker and study its value for clinical practice for diagnosis of MS subtypes and surrogate endpoint of disease progression in MS.

METHODS: In this ongoing study we have so far analysed serum samples from 90 MS patients (relapsing remitting (RRMS) n=50, primary progressive (PPMS) n=40). 24 healthy volunteers (HC) served as controls. For detection we used polyclonal antisera obtained from rabbits previously immunized with the SGIII peptide Cys-KPGGSQDKSLHNRELSAERPLNEQIAEAEED-A. All analyses were performed by Western Blotting. The presence and intensity of the different bands were rated in a semi-quantitative and quantitative manner.

RESULTS: Several bands positive for the anti-SGIII antibodies were observed, supposed to be isoforms, aggregates and fragments. The SGIII positive bands of 12 kD, 23 kD, 37 kD and 100 kD discriminated MS patients from controls (p<0.01). SGIII isoform at 37 kD discriminated PPMS patients from RRMS patients (p<0.05). Considering the entire patient cohort, band intensities at 37 kD positively correlated with physical disability (R=0.238, p<0.05). Untreated MS patients had significantly higher band intensities at 23 kD (p<0.01) and 100 kD (p<0.05) compared to patients receiving immunomodulatory treatment.

CONCLUSION: The present study provides evidence that specific isoforms of serum SGIII may serve as a supporting tool to discriminate MS subtypes, especially PPMS patients. Furthermore, assessment of SGIII isoforms might provide a tool to monitor disease progression and treatment effects in MS.
INTRODUCTION

Multiple sclerosis (MS) is a chronic neurological disease in which immune-mediated axonal loss closely correlates with disability (Bjartmar, et al. 2000). Most of the patients (70-80%) start with a relapsing remitting (RRMS) disease course, subsequently converting to a secondary progressive phase (SPMS) in which irreversible disability increases gradually (Noseworthy, et al. 2000). Only a few patients start with a primary progressive MS (PPMS) subtype which accounts for 10-15% of the MS patients, characterised by progressive disability in the absence of relapses or recovery (Noseworthy, et al. 2000).

There is a strong need for blood biomarkers to stratify patients into subtypes, which is relevant for treatment decisions (Bielekova and Martin. 2004). Besides assessment of the IgG-index and oligoclonal IgG bands in CSF for diagnostic purposes and neutralizing antibodies directed against interferon-beta to monitor treatment efficacy (Polman, et al. 2010), no other biomarkers are available in clinical practice for MS (Teunissen, et al. 2005, Tumani, et al. 2009). Therefore, novel biomarkers are strongly needed. Development of serum biomarkers for disease sub-typing would be extremely useful in two respects: 1) the biomarkers would allow treatment decisions, as the majority of treatments are only effective in RRMS patients and not in PPMS patients, 2) the biomarker(s) would allow development of therapeutic drugs designed for the different clinical subtypes.

In a recent proteomics-study, we have identified that a fragment of secretogranin III (SGIII) in serum might be a potential candidate biomarker for MS (Teunissen, et al. 2011). Secretogranin is a member of granin protein family that are involved in secretion of molecules by cells (Taupenot, et al. 2003). The potential value of granins as biomarkers for neuropsychiatric disorders has recently gained much interest (Bartolomucci, et al. 2010). Due to their secretion into the CSF, saliva and blood, assessment of granins provides a promising tool to establishing biomarkers for various disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer’s disease, frontotemporal dementia, and schizophrenia (Bartolomucci, et al. 2010). SGIII is primarily expressed in neuroendocrine and nervous tissue, for example in cells of the hypothalamus-pituitary axis. In vivo studies show that several CNS cell types produce and transport SGIII (Helle. 2004). In particular, the highest SGIII expression in the cerebral cortex in vivo is present in astroglial cells, which increase dramatically upon neuronal damage (Paco, et al. 2010). Furthermore, various studies showed altered expression of granins in patients with disorders involving cognitive impairment (Bartolomucci, et al. 2010).

So far SGIII has not been investigates in MS. We therefore aimed to evaluate the potential use of serum SGIII for diagnosis and disease sub-typing in MS and whether physical disability, cognitive performance and MS therapy are related to serum SGIII levels.

SUBJECTS / METHODS

Patients and controls

Baseline serum samples were obtained between 2000 and 2004 and stored at ~80 °C until use. All patients underwent detailed clinical examination and clinical data documented included age, age at disease onset, disease duration, Expanded Disability Status Scale (EDSS) (Kurtzke. 1983), and the Multiple Sclerosis Functional Composite Score (MSFC) (Cutter, et al. 1999). The
MSFC consisted of 3 subtests, including the nine-hole peg test, timed walk (walk of 25 ft) and the Paced Auditory Serial Addition Test (PASAT). Information on treatment was available in 46 patients, amongst them 29 patients received MS treatment (Interferon beta 26, glatiramer acetate 2, methotrexate 1). Detailed clinical and demographical data are given in Table 1. IRB-approval was obtained, and all patients signed an informed consent.

**Anti secretogranin III antibodies**

For secretogranin III (SGIII) detection, Biogenes (Berlin, Germany) developed polyclonal anti sera from rabbits and mice immunized with the SGIII fragment peptide Cys-KPGGSQDKSLHNRE LSAERPNEQIAEAEED-A. All analyses were performed by Western Blot.

**Western Blotting**

Western Blot analyses were performed according to standard methods. Briefly, 0.5 µl serum was loaded per slot using 4-12% Bis-Tris gels 1.0 mm X 15 well (NuPage, Invitrogen, Carlsbad, CA, USA). Separated proteins were then electrotransferred to PVDF membranes (Immobilon, Millipore, Tullagreen, Carrigtwohill, Cork, Ireland). Blots were then blocked for 30 min with 5% milk powder dissolved in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). Primary rabbit polyclonal anti-SGIII antibody (Biogenes, Berlin, Germany), diluted 1:10000 in 5% milk powder dissolved in PBS containing 0.05% Tween 20 PBS-T, was added to blot membranes for incubation at room temperature overnight on a shaker. Blots were then washed 3 times 10 min in PBS containing 0.05% Tween 20 (PBS-T) supplemented with 0.5% milk powder. For detection, blots were incubated with horseradish peroxidase (HRP) labelled polyclonal swine anti rabbit immunoglobulins (DAKO, Glostrup, Denmark) for 30 min at room temperature on a shaker. After washing again 3 times 10 min with 0.5% milk powder dissolved in PBS containing 0.05% Tween 20, blots were then developed with ECL (Amersham, Little Chalfont, UK) and visualized on Kodak films (Sigma-Aldrich, St. Luis, USA). Intensities of SGIII isoforms were then rated in a semi-quantitative and blinded manner.

For quantitative analyses blots were redeveloped with a fluorescence labelled antibody according to the following protocol. After washing 6 times 10 min with PBS-T 0.05% at room temperature, blots were incubated with fluorescently labelled secondary antibody for 30 min at room temperature and visualized under a fluorescence microscope (Carl Zeiss, Jena, Germany).

**Table 1. Clinical and demographic data**

<table>
<thead>
<tr>
<th></th>
<th>RRMS</th>
<th>PPMS</th>
<th>HC</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>N (% female)</td>
<td>50 (60)</td>
<td>40 (55)</td>
<td>24 (63)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age baseline (yrs)</td>
<td>39.1 (9.5)</td>
<td>53.8 (11.7)</td>
<td>37.3 (9.7)</td>
<td>PPMS vs RRMS and HC p&lt;0.001</td>
</tr>
<tr>
<td>Age at disease onset (yrs)</td>
<td>32.7 (9.8)</td>
<td>39.2 (10.9)</td>
<td>NA</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Disease duration (yrs)</td>
<td>6.1 (3.6-8.9)</td>
<td>13.8 (6.4-19.2)</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EDSS *</td>
<td>3.0 (2.0-4.0)</td>
<td>6.0 (4.5-7.5)</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MSFC *</td>
<td>0.4 (0.2-0.8)</td>
<td>-0.4 (-1.1-0.2)</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
</tbody>
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N, number of patients/controls; RRMS, relapsing remitting MS; PPMS, primary progressive MS; HC, healthy controls; yrs, years; n.s., statistically not significant; EDSS, expanded disability status scale; MSFC, multiple sclerosis functional composite score; NA, not applicable. Values are presented as number (percent), mean (SD) or as * median (interquartile range).
temperature on a shaker, blots were incubated with biotinylated polyclonal swine anti rabbit immunoglobulins (DAKO (product number: E0431), Glostrup, Denmark) diluted in Odyssey blocking buffer (LI-COR Biosciences, Bad Homburg, Germany) 1:1 with PBS-T for 1 hour at room temperature on a shaker. Blots were then washed again 6 times 10 min with PBS-T at room temperature on a shaker and subsequently incubated with IRDye® 680 Streptavidin (LI-COR Biosciences, IRDye® 680 Streptavidin, product number: 926-32231, Bad Homburg, Germany) for 45 minutes at room temperature on a shaker and in the dark. Thereafter, blots were washed 4 times 10 min with PBS-T followed by 4 times 10 min with PBS (all at room temperature on a shaker in the dark). Western Blot band intensities were then quantified using Odyssey infrared imaging software (LI-COR Biosciences, Bad Homburg, Germany).

Statistical analysis
Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, Illinois, USA). Gaussian distribution of data was tested by the Kolmogorov–Smirnov test. Comparisons between groups were performed by Mann–Whitney U test. In the combined patient groups, Spearman and Pearson correlations were performed to calculate the correlation coefficients between Western Blot band intensities with clinical and demographical data. ROC analysis was applied to determine if SGIII isoforms discriminated MS patients from controls. Pearson partial correlations were performed on ranked variables to correct for age.

RESULTS
Comparison of Western Blot SGIII intensities between MS and HC
Figure 1 shows a representative Western Blot picture. Statistical significant SGIII isoform group differences between MS and controls were present at 12 kD, 23 kD, 37 kD and 100 kD. In MS, band intensities at 12 kD, 23 kD and 37 kD were significantly increased (p<0.01), whereas 100 kD isoform intensity was significantly decreased (p<0.01) compared to controls (Figure 2).

When considering RRMS patients only, band intensities at 12 kD and 23 kD were significantly increased (p<0.05) and 100 kD was significantly decreased (p<0.005) compared to controls. In PPMS patients, significant increased band intensities compared to controls were present at 12 kD (p<0.01), 23 kD (p<0.001) and 37 kD (p<0.005). In contrast to RRMS, significantly decreased band intensity at 100 kD was not present in PPMS patients.

When comparing MS subgroups, PPMS patients had significantly higher band intensities at 37 kD (p<0.05) compared to RRMS. Figures 3 A-D summarize subgroup analyses of SGIII band intensities.

Correlation of Western Blot SGIII intensities with clinical and demographical data
We found a correlation of band intensities at 37 kD with increasing age (R=0.262; p<0.05) and with age at disease onset (R=0.225; p<0.05). Considering the entire patients cohort, EDSS values positively correlated with band intensities at 37 kD (R=0.319; p<0.005) and 100 kD (R=0.263; p<0.05). This correlation remained significant for band intensities at 37 kD (R=0.238, p<0.05) after adjusting for age.
Figure 1. Fluorescence stained SGIII Western Blot. Detection of distinct reactivity patterns by polyclonal anti secretogranin III antibody in different serum samples of MS patients. Each lane corresponds to one serum sample.

Figure 2. ROC analysis of SGIII isoforms in MS and controls. SGIII isoforms at 12 kD, 23 kD and 37 kD were significantly increased (p<0.01) in MS compared to controls, whereas 100 kD isoform intensities were significantly decreased in MS (p<0.01).

Baseline multiple sclerosis functional composite score (MSFC) values were weakly to moderately and negatively correlated to band intensities at 37 kD (R=-0.320; p<0.005) and 100kD (R=-0.234; p<0.05). We then explored whether cognitive performance, assessed by the PASAT, which is part of the MSFC were specifically related to the band intensities of these two isoforms. No significant correlations emerged. Furthermore, no significant correlation was present between SGIII isoforms and disease duration. There was no significant gender difference of SGIII band intensities.

Influence of MS treatment on Western Blot SGIII intensities

We then investigated whether MS therapy influenced Western Blot SGIII isoform intensities. Untreated MS patients showed significantly higher band intensities at 23 kD (p<0.01) and 100 kD (p<0.05) compared to patients receiving MS therapy. Figure 4 displays group differences for 23 kD intensities in treated and untreated patients.
Figure 3 A-D. Subgroup analyses of SGIII band intensities

Figure 3 A. SGIII isoform Western Blot band intensities at molecular weight of 12 kD. Significant higher band intensities are present in RRMS and PPMS compared to controls. RRMS – relapsing remitting multiple sclerosis, PPMS – primary progressive multiple sclerosis. Horizontal bars represent medians.

Figure 3 B. SGIII isoform Western Blot band intensities at molecular weight of 23 kD. Significant higher band intensities are present in RRMS and PPMS compared to controls. RRMS – relapsing remitting multiple sclerosis, PPMS – primary progressive multiple sclerosis. Horizontal bars represent medians.

Figure 3 C. SGIII isoform Western Blot band intensities at molecular weight of 37 kD. Significant higher band intensities are present in PPMS compared to RRMS and controls. RRMS – relapsing remitting multiple sclerosis, PPMS – primary progressive multiple sclerosis. Horizontal bars represent medians.

Figure 3 D. SGIII isoform Western Blot band intensities at molecular weight of 100 kD. Significant decreased band intensities are present in RRMS compared to controls. RRMS – relapsing remitting multiple sclerosis, PPMS – primary progressive multiple sclerosis.

Figure 3 A-D. Subgroup analyses of SGIII band intensities
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DISCUSSION

In a recently performed proteomics study we identified serum secretogranin III (SGIII) as a potential candidate biomarker in MS (Teunissen, et al. 2011). The aim of this ongoing study was to evaluate the potential use of serum SGIII for disease sub-typing in MS.

In the initial proteomics study on MS patients’ serum we found decreased levels of a SGIII fragment, which was below 10 kD (Teunissen, et al. 2011). This is supported by other recent publications showing decreased secretogranin levels in MS (Mattsson, et al. 2007, Stoop, et al. 2008). However, the SGIII isoforms detected by Western Blotting of the present study were higher than that one identified in the initial proteomic based study (Teunissen, et al. 2011). Moreover, we did not detect the fragment of 3kD in MS serum. We do not have an explanation for this. Aggregation of SGIII fragments in serum samples and during Western Blot procedures could be the underlying reason for our finding. In addition we found different patterns of Western Blot band intensities in MS patients compared to controls. While Western Blot band intensities at 100 kD were significantly decreased, three other SGIII isoforms (12 kD, 23 kD, 37 kD) were significantly increased in MS compared to controls. Due to the fact that some SGIII isoforms are elevated in MS whereas others are not, it is important to analyze distinct SGIII patterns rather than the whole signal. When comparing PPMS with RRMS patients we found increased Western Blot signals in PPMS at 37 kD. Analyzing serum SGIII isoforms could thus provide a supporting tool for diagnosing MS subtypes. Especially in patients with PPMS the time lag between disease onset and diagnosis (Polman, et al. 2011) takes often longer than in the relapsing remitting form.

Western Blot SGIII band intensities at 37 kD correlated with increasing age. Thus, it is important to correct for age, when performing statistical analysis, to ensure adequate clinical interpretations. After correcting for age, band intensities at 37 kD still correlated with the EDSS score at time of blood sampling. In addition, 23 kD and 100 kD bands of SGIII were significantly lower in patients receiving immunomodulatory treatment compared to untreated patients. Assessment of SGIII isoforms could thus also serve as a potential biomarker to monitor treatment response.
Another interesting finding is the correlation of SGIII isoforms with the multiple sclerosis functional composite score (MSFC) (Cutter, et al. 1999), a suggested outcome measure for clinical trials. The MSFC comprises tests for ambulation of major clinical dimensions of arm, leg and cognitive function. One measure of the MSFC is the Paced Auditory Serial Addition Test (PASAT), testing performance on attention, working memory, and information processing speed, which are frequently impaired in MS (Chiaravalli and DeLuca. 2008). Independent from physical disability, cognitive impairment significantly affects the patients’ quality of life and impacts employment and social functioning (Bobholz and Rao. 2003, Rao, et al. 1991).

CSF levels of granin family members have been shown to be altered in disorders affecting cognition (Bartolomucci, et al. 2010). However, when analyzing SGIII band intensities and PASAT scores, no significant correlation was found. This indicates that changes in serum SGIII isoforms occur independently from cognitive performance regarding the cognitive domain of attention, working memory, and information processing speed. To investigate whether serum SGIII isoforms might be related to performance in other cognitive domains, a comprehensive neuropsychologic test battery, e.g. the Biref Repeatable Battery of Neuropsychological tests (BRB-N) (Boringa, et al. 2001), should be used in future studies.

SGIII belongs to the granins family consisting of uniquely acidic proteins of the diffuse neuroendocrine system (Helle. 2004). Granins exert both intracellular and extracellular functions (Helle. 2004). Intracellularly, they are important for protein trafficking (Taupenot, et al. 2003), while extracellular they may serve as pro-hormones for putative regulatory peptides either locally or distinct from the site of secretion (Helle. 2004). However, up to now only little is known about functional properties of SGIII derived peptides (Helle. 2004).

Our present findings in conjunction with the initial proteomic study (Teunissen, et al. 2011) suggest that immunopathological processes during the course of MS may lead to differential expression and secretion of SGIII peptides into the CSF and blood (Bartolomucci, et al. 2010). Furthermore, the finding that immunomodulatory treatment has an impact on serum SGIII isoform patterns also supports the notion of a possible close relation between immunologic conditions and serum SGIII peptides levels.

Up to now, besides extensive efforts, no blood biomarkers are available indicating disease progression in MS (Teunissen, et al. 2005). Having an easy accessible paraclinical parameter related to advancing MS would facilitate patients management and treatment decisions in MS. Towards this end, the present preliminary and ongoing study provides evidence that specific isoforms of SGIII might be used as biomarkers for sub-typing MS patients, especially to define PPMS patients and to monitor disease progression and treatment effects. Future research should focus on the development of quantitative assay systems, e.g. based on ELISA or multiparameter immunoassays. Since some SGIII isoforms are diversely regulated in MS and controls separate detection of distinct isoforms would be a requirement for such immunoassays.

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