Serum Glial Fibrillary Acidic Protein as a Diagnostic Biomarker in Dogs with Progressive myelomalacia

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Running head: THE BIOMARKER FOR PROGRESSIVE MYELOMALACIA IN DOG
ABSTRACT. In humans, increased levels of GFAP in the CSF and blood have been reported with various neural diseases. However, there has been no study describing the usefulness of GFAP in the blood for disease of the spinal cord in dogs. To describe the utility of GFAP in serum for a diagnosis of progressive myelomalacia. Fifty-six dogs with acute thoracolumbar IVDD diagnosed by computed tomography with myelography or MRI were included. Serum specimens were collected at initial presentation from all cases and at follow-up examinations from some cases. Serum samples were assayed for GFAP concentrations using a commercially available GFAP ELISA KIT. Progressive myelomalacia was the final diagnosis in 8/52 (15.3%). Eight dogs had clinical signs suggestive of progressive myelomalacia, of which 6 were positive and 2 were negative by GFAP. Seven dogs had a detectable level of serum GFAP, of which 6 had the onset of progressive myelomalacia. The sensitivity and specificity of the GFAP to progressive myelomalacia were 75% and 97.7%, respectively. The results suggest the utility of GFAP in serum in the diagnosis of progressive myelomalacia.

KEY WORDS: glia, Neurology, diagnosis, Dog
INTRODUCTION. Progressive myelomalacia is thought to result in ischemic and hemorrhagic necrosis of the spinal cord, which can occur following acute spinal cord injury, and represents extensive damage of the intramedullary spinal vasculature [7, 10,15]. Although the exact pathophysiological mechanism of myelomalacia is poorly understood, it seems to be the result of the concussive effects of trauma followed by ischemia, which results in release of vasoactive substances, oxygen-free radicals and cellular enzymes [15]. Progressive myelomalacia frequently results from thoracolumbar intervertebral disc disease (IVDD) in dogs [6,8,13,18]. The prognosis of thoracolumbar IVDD is good if the treatment is initiated early in the course of the disease [6, 8]. However, when the onset of progressive myelomalacia is suspected, it is necessary to suspend surgical treatment. Though the possibility of the cutaneous trunci muscle reflex for diagnosing progressive myelomalacia was suggested in previous report [1], clinical signs and abnormal findings of neurological examination of progressive myelomalacia take several days to appear after the development of paraplegia [13,15]. There is currently no effective way to detect progressive myelomalacia at the early-stage. Recently, diagnostic imaging such as myelography and
MRI have been used to attempt diagnosis of progressive myelomalacia, but the accuracy was not sufficient \([12,15]\). Nevertheless, differentiation of progressive myelomalacia from IVDD is of clinical importance for the choice of appropriate treatment.

Glial fibrillary acidic protein (GFAP) is the major protein constituent of glial filaments in differentiated astrocytes of the central nervous system \([2]\). Astrocytes are an important cellular component for maintenance of the blood brain barrier \([9, 4]\), and to modulate the efficacy of synapses \([11]\). GFAP is tightly packed into intermediate filament polymers and is generally insoluble \([14]\). However, when astrocytes are damaged, GFAP is released into the cerebrospinal fluid (CSF) as soluble fragments \([8, 10]\). In humans, increased levels of GFAP in the CSF and blood have been reported with various neural diseases, for example systemic lupus erythematosus, multiple sclerosis, Alzheimer’s disease, subarachnoid hemorrhage and traumatic brain injury \([5,19,20]\). We postulated that GFAP may be released to peripheral blood in progressive myelomalacia following neural damage. However, we were unable to find any studies that show the usefulness of GFAP for disease of the spinal cord in dogs. This study describes the utility of GFAP in the blood for an early diagnosis of progressive
myelomalacia.
MATERIALS AND METHODS. The databases of the Animal Hospital of Iwate University and Maizuru Animal Medical Center were reviewed for cases of canine IVDD from April to November in 2011. Of a total of 97 cases with a diagnosis of IVDD with diagnostic imaging, computed tomography and myelography or MRI, 51 were selected for this study on the basis of the following case definition: Herniation was located in lumbar vertebra and hindlimb paralysis was presented. After diagnosis, operations were performed exclusive of six case of progressive myelomalacia. The decompression by the extruded disk material was confirmed at all operated case. Follow-up evaluation was performed until three month after operation. The dogs that had not made a successful recovery by discharge were contacted again to determine whether they had recovered in actual examination.

Neurological examination

All dogs had a neurological examination at presentation, and a grade was recorded in the medical records: grade 1 – spinal pain; grade 2 – ambulatory paraparesis, and/ or ataxia and/ or proprioceptive deficits; grade 3 – non-ambulatory paraparesis; grade 4 –
paraplegia with intact deep-pain perception and grade 5 – paraplegia with no deep-pain perception.

Collection of serum

Serum specimens were collected from all cases at first medical examination and unscheduled sampling was performed in some cases. The specimens were centrifuged at 12,000 × g for 2 min at 4 °C to remove the cells, and the supernatant was stored at -80 °C until biomarker assays were performed. All samples were prepared within 30 minutes.

GFAP assay for the serum

Serum samples were assayed for GFAP using a commercially available GFAP ELISA system (BioVendor Laboratory Medicine, Candler, NC). The samples were assayed according to the protocol provided by the kit manufacturer. Briefly, 100 μL of the prepared standard and 100 μL of each sample were incubated at 25 °C for 2 h. The wells were then aspirated, washed, and incubated with 100 μL of the Biotin labeled antibody at 25 °C for 1 h. Next, the wells were aspirated, washed, and incubated with 100 μL of
the streptavidin-HRP conjugate solution at 25 °C 1 h. Then, the wells were aspirated, washed, and incubated with 100 μL of the substrate solution at room temperature for 10-15 min. This reaction was stopped by the addition of 100 μL of stop solution and the optical density was measured at 450 nm using a spectrophotometer (Tosoh Corporation, Tokyo, Japan). The concentrations of GFAP were determined according to a standard curve and reported in ng/ml.

Statistical analysis

Sensitivity, specificity, positive predicted value, and negative predicted value compared to the final diagnosis of progressive myelomalacia were calculated for the serum GFAP test. Logistic regression was used to calculate relative risk and ninety-five percent confidence intervals (95% CI) summarizing the association between the onset of progressive myelomalacia and serum GFAP. A $P$-value of 0.05 or less was considered statistically significant.
RESULTS. A total of 51 dogs fulfilled the incision criteria. Seventy-nine percent (41/52) were Dachshunds, and 82% (43/52) were chondrodystrophoid (Table 2). Dogs were aged 4-14 years (mean 6.5 years).

Progressive myelomalacia was the final diagnosis in 8/52 (15.3%) cases with characteristic symptom as follows: flaccid paraplegia, total areflexia of the pelvic limbs, tail and anus, loss of deep pain perception caudal to the site of spinal cord injury, flaccid abdominal musculature, depressed mental state, and respiratory difficulty due to intercostals and diaphragmatic paralysis. Any marked findings suggested progressive myelomalacia were not found using diagnostic images. While the remaining cases improved in clinical signs regardless of whether the surgical treatment was performed,

Intraoperative aspect suspected progressive myelomalacia was found in one case (case 42) that was not detected serum GFAP at first visit. At last, the serum GFAP of this case was never evaluated again. Serum GFAP of Case 37 was detected two days after developing paraplegia. Four days later the dog was euthanatized in the cause of the development of progressive myelomalacia. Similarly, six of 8 dogs onset progressive myelomalacia showed a positive for serum GFAP at two days after developing paraplegia.

The symptoms with regard to progressive myelomalacia were gradually progressed
toward the head in all cases (Table 3). In this research, cranial movement of the border
of the CTM reflex, lower motor neuron sign in the forelimb, respiratory disturbance and
horner syndrome were occurring by turns in most dogs. This information matches a past
report. Especially, cranial movement of CTM reflex has a strong association with
progressive myelomalacia in the previous report [1]. At last, all dogs developed
progressive sign consisted with progressive myelomalacia were died (6 with nature
death and 2 with euthanasia). The period from paraplegia onset to dead was 6.8 (3-12)
days.

8 dogs showed the onset of progressive myelomalacia, of which 6 were positive and 2
were negative by GFAP. In 7 dogs, serum GFAP was detected, of which 6 showed the
onset of progressive myelomalacia. The sensitivity and specificity of the GFAP to
progressive myelomalacia were 75% and 97.7%, respectively. A significant association
between progressive myelomalacia and serum GFAP was shown (RR=18.857 (4.709,
75.514)) (table 3).

Of 43 dogs that were not developed progressive myelomalacia, 3 dogs were not
evaluated postoperative recovery after discharge. Follow-up evaluation was performed
on the remaining 40 dogs for 3 months postoperatively. All 32 dogs grouped grade III
and IV showed an improvement in motor function until three months after operation.
Eight of the 11 dogs that were classified as the grade V were performed postoperative
examination; 4 dogs recovered motor function, One dog recovered nociception, and 3
dogs were not showed improvement until 3 months after operation.
DISCUSSION. To the best of our knowledge, this is the first study on the biomarker of progressive myelomalacia in the blood.

IVDD is commonly encountered in small animal practice. As a consequence of advances in diagnosis and treatment for it, the outcome can be stabilized. In fact, all dogs besides the case of progressive myelomalacia in this study showed improved symptoms by treatment. However, there is poor outcome in progressive myelomalacia regardless of therapy, because its lesion is thought to be irreversible and progressive.

Thereby, an index is needed to indicate whether myelomalacia is progressive for adequate prediction. The cranial movement of the cutaneous trunci reflex indicated of progressive myelomalacia approximately takes about 5-7 days after the appearance of paraplegia [13]. In fact, GFAP was detected in serum less than 5 days from the dogs affected with progressive myelomalacia in this study. And GFAP was detected in serum earlier than the onset of highly characteristic clinical presentation of progressive myelomalacia. These results support the usefulness of GFAP in serum for an early diagnosis of progressive myelomalacia.

The sensitivity and specificity of GFAP to differentiate progressive myelomalacia
shown in this study are thought to be sufficient. However, GFAP was negative in 2 dogs with progressive myelomalacia. In the event of injuring the spinal cord, a time lag might be needed before GFAP appears in the serum from the affected area of the spinal cord. In both cases, GFAP in the serum was measured at only the first visit; thereby it might be undetected in serum. The timing of blood sampling from the appearance of paraplegia may be important for the opportunity to measure GFAP in the serum for prediction of progressive myelomalacia. Therefore it might be better to test of GFAP repeatedly, if it doesn’t appear at first meeting. In fact, a dog that was GFAP-negative at the first time indicated GFAP in the serum at second test. Although, in this study, the time course of GFAP appearing in the blood was unclear, it was suggested that at least twenty-four hours need to detect GFAP in the serum from advancing to paralysis. Because, in all cases, GFAP appeared in blood was 24 hour after paralysis onset. If the 2 cases undetected of GFAP measured in serum not only first-visit but also over time, GFAP might have been detected. As a result, the sensitivity of GFAP for progressive myelomalacia may indicate a higher value compared to that in this study. GFAP was detected in 1 case of thoracolumbar IVDD without progressive
myelomalacia in this study. Any marked findings of diagnostic images were not found in this dog. In the end, this case remained nonambulatory paraparetic at 3 months after the operation. On the other hand, in the dogs classified as the grade III and IV, GFAP in the serum was not appeared and postoperative outcomes were successful. These results suggested a correlation between detecting GFAP and the severity of spinal cord lesion. The concentrations of GFAP in the serum of the dog with progressive myelomalacia were significantly higher than those of the dogs with thoracolumbar IVDD without it and those of the healthy dogs. This suggests the possibility that astrocytes were more severely damaged in myelomalacia than thoracolumbar IVDD. As the impact of injury may be an important factor in the progression of myelomalacia, the low value indicated in IVDD reflected that the lesion was mild. The other possibility is that there are two types of pathological status in myelomalacia. The first type is progressive myelomalacia. Progressive myelomalacia is thought to be ischemic or hemorrhagic necrosis of the spinal cord that can occur following acute spinal cord injury and represents extensive damage of the intramedullary spinal vasculature. Dogs that develop progressive myelomalacia quickly die. The second type is a focal lesion.
This type of lesion area was confined to a minimum without expanding, so the possibility of recovery might be expected [6, 13]. It is difficult to distinguish between thoracolumbar IVDD and focal lesion type of myelomalacia. This type of lesion is the reason why one dog without developing progressive myelomalacia showed GFAP in the blood in this research. The pathway of GFAP in CSF leak into systemic blood was not investigated in this study. However, two possibilities were speculated about the pathway. The first possibility is destruction of the blood-brain barrier. GFAP is the principal intermediate filament in mature astrocytes of the central nervous system [2]. Astrocytes are involved in the integrity of the blood brain barrier [4, 9] so when the BBB breaks down in consequence of astrocytes damaged by progressive myelomalacia, GFAP is able to be detected in the blood. The second possibility is GFAP appear in the blood by concentration dependence. In dogs with progressive myelomalacia, the concentrations of GFAP in CSF may be very high, resulting in leakage into the blood. This hypothesis might be shown by examination of GFAP correlated within CSF and the blood. Unfortunately, GFAP in CSF was not measured in this study. A definite diagnosis of progressive myelomalacia should be based on pathological
examination, however sampling a biopsy specimen from the spinal cord while alive is impossible. Consequently, in the clinical setting, progressive myelomalacia is diagnosed based on the characteristic clinical presentation as follows; flaccid paraplegia, total areflexia of the pelvic limbs, tail and anus, loss of deep pain perception caudal to the site of spinal cord injury, flaccid abdominal musculature, depressed mental state, and respiratory difficulty due to intercostals and diaphragmatic paralysis. The same diagnosis was used in the current study.

In the current study, GFAP was measured by using a commercial ELISA kit for human GFAP. This kit was used in a previous study for measuring the GFAP of dogs. To validate the availability of the ELISA kit for dogs, the cross reactivities of anti-human GFAP antibodies in the kit with canine GFAP were confirmed by an indirect immunofluorescence assay using cultured canine astrocytes [19].

In this study, the efficacy of GFAP in the blood as a predictive factor for progressive myelomalacia was demonstrated by the strong correlation. This outcome will contribute to deciding whether surgical repair is applied for paralysis.

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Table 1: GFAP value of dogs in each grade.

Data are presented number, detection of GFAP and blood concentration of GFAP.

Table 2: process of the symptoms of progressive myelomalacia.

A day developing paraplegia is defined as zero day.

Table 3: RR, sensitivity and specificity of GFAP for progressive myelomalacia.
<table>
<thead>
<tr>
<th>Grade</th>
<th>number</th>
<th>GFAP</th>
<th>GFAP value (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>not detected</td>
<td>detected</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>22</td>
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<td></td>
<td>+</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>case</td>
<td>serum GFAP</td>
<td>specific symptom of MP and its onset day</td>
<td></td>
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<tr>
<td>------</td>
<td>-----------------</td>
<td>-----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>negative at 2day</td>
<td>6day horner syndrome euthanasia</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>positive at 2day</td>
<td>4day protrusion of the nictitating membrane&lt;br&gt;5day euthanasia</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>positive at 2day</td>
<td>4day protrusion of the nictitating membrane&lt;br&gt;10day respiratory disturbance&lt;br&gt;10day natural death</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>negative at 2day</td>
<td>unknown&lt;br&gt;12day natural death</td>
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<tr>
<td>45</td>
<td>positive at 2day</td>
<td>3day cranial movement of the border of the CTM reflex&lt;br&gt;lower motor neuron sign in the forelimb&lt;br&gt;horner syndrome&lt;br&gt;respiratory disturbance&lt;br&gt;4day natural death</td>
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<tr>
<td>47</td>
<td>positive at 2day</td>
<td>3day cranial movement of the border of the CTM reflex&lt;br&gt;lower motor neuron sign in the forelimb&lt;br&gt;horner syndrome&lt;br&gt;respiratory disturbance&lt;br&gt;4day natural death</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>negative at 1day&lt;br&gt;positive at 3day</td>
<td>7day cranial movement of the border of the CTM reflex&lt;br&gt;lower motor neuron sign in the forelimb&lt;br&gt;horner syndrome&lt;br&gt;respiratory disturbance&lt;br&gt;8day natural death</td>
<td></td>
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<tr>
<td>49</td>
<td>negative at 2day&lt;br&gt;positive at 4day</td>
<td>5day cranial movement of the border of the CTM reflex&lt;br&gt;lower motor neuron sign in the forelimb&lt;br&gt;horner syndrome&lt;br&gt;respiratory disturbance&lt;br&gt;6day natural death</td>
<td></td>
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<tr>
<td>GFAP</td>
<td>Progressive Myelomalacia</td>
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<tr>
<td></td>
<td>onset</td>
<td>not-onset</td>
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<td>Not-detected</td>
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<td>Relative Risk</td>
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<td>4.709 - 75.514</td>
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<tr>
<td>Sensitivity</td>
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<td>0.483 - 0.850</td>
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<tr>
<td>Specificity</td>
<td>0.977</td>
<td>0.927 - 0.995</td>
<td></td>
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RR, sensitivity and specificity of GFAP for progressive myelomalacia.