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**Histidine-rich Glycoprotein Could Be an Early Predictor of
Vasospasm after Aneurysmal Subarachnoid Hemorrhage**

香川大学大学院医学系研究科

機能構築医学

松 本 淳 志

**Histidine-rich Glycoprotein Could Be an Early Predictor of Vasospasm after
Aneurysmal Subarachnoid Hemorrhage**

Atsushi Matsumoto^a, Takehiro Nakamura^{b*}, Aya Shinomiyaa, Kenya Kawakitac,

Masahiko Kawanishia, Keisuke Miyakea, Yasuhiro Kurodac,

Richard F. Keep^d, Takashi Tamiyaa

松本淳志, 中村丈洋, 四宮あや, 河北賢哉, 川西正彦, 三宅啓介,

黒田泰弘, Richard F. Keep, 田宮隆

^aDepartment of Neurological Surgery, Kagawa University Faculty of Medicine, Miki,

Kagawa 761-0193, Japan

^bDepartment of Medical Technology, Kagawa Prefectural University of Health Sciences,

Takamatsu, Kagawa 761-0123, Japan

^cEmergency Medical Center, Kagawa University Hospital, Miki, Kagawa 761-0793,

Japan

^dDepartment of Neurosurgery, University of Michigan, Ann Arbor, MI 48109, USA

Running title: A New Biomarker for SAH: HRG

*Corresponding author : Takehiro Nakamura, 中村丈洋

Department of Medical Technology, Kagawa Prefectural University of Health Sciences

281-1 Hara, Mure, Takamatsu, Kagawa 761-0123, Japan.

TEL: +81-87-870-1212, FAX: +81-87-870-1202, E-mail: tanakamu@kms.ac.jp

ABSTRACT

Cerebral vasospasm (CVS) is a major contributor to the high morbidity and mortality of aneurysmal subarachnoid hemorrhage (aSAH) patients. We measured histidine-rich glycoprotein (HRG), a new biomarker of aSAH, in cerebrospinal fluid (CSF) to investigate whether HRG might be an early predictor of CVS. A total of seven controls and 14 aSAH patients (eight males, six females aged 53.4 ± 15.4 years) were enrolled, and serial CSF and serum samples were taken. We allocated these samples to three phases (T1–T3) and measured HRG, interleukin (IL)-6, fibrinopeptide A (FpA), and 8-hydroxy-2'-deoxyguanosine (8OHdG) in the CSF, and the HRG in serum. We also examined the release of HRG in rat blood incubated in artificial CSF. In contrast to the other biomarkers examined, the change in the CSF HRG concentration was significantly different between the non-spasm and spasm groups ($p < 0.01$). The rat blood/CSF model revealed a time course similar to that of the human CSF samples in the non-spasm group. HRG thus appears to have the potential to become an early predictor of CVS. In addition, the interaction of HRG with IL-6, FpA, and 8OHdG may form the pathology of CVS.

Key words

biomarker, histidine-rich glycoprotein, predictor, subarachnoid hemorrhage, vasospasm

Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) is a devastating type of stroke with high morbidity and mortality. The mortality is approx. 60%, with 30% of survivors having significant morbidity. Cerebral vasospasm (CVS) and microvascular dysfunction associated with aSAH can decrease the cerebral blood flow, leading to delayed cerebral ischemia (DCI) and hypoxia in brain tissues and ultimately to severe neurological dysfunction [1]. These are major contributors to the high morbidity and mortality of aSAH patients. A CVS usually occurs at around 4 days after the onset of aSAH and reaches a peak in incidence and severity at 7–10 days [2].

There is substantial evidence to suggest that oxidative stress, inflammation, alterations in the coagulation system, and subsequent vascular endothelial injury are significant in the development of acute brain injury and CVS following aSAH. The pathogenesis of CVS has not been fully clarified. Various cytokine and protein profiles in serum or CSF have been investigated to elucidate this pathogenesis. Although some biomarkers have shown a relationship with CVS, an effective early predictor has not been found. In addition, the interactions between biomarkers are still unknown.

Histidine-rich glycoprotein (HRG) is a plasma glycoprotein produced in and secreted from the liver. Since HRG binds to a diverse range of ligands, it has been

suggested to be involved in the regulation of coagulation/fibrinolysis [3–5], immune response [6], and angiogenesis [7–10]. HRG was also recently shown to have important effects on intravascular elements. HRG inhibits the tight attachment of neutrophils, sustains rheological stability, and inhibits vascular endothelial cell activation [11,12]. This suggests that a loss of HRG might be involved in the endothelial injury that leads to a CVS. However, HRG in cerebrospinal fluid (CSF) has not been investigated.

The primary aims of the present retrospective study were to determine whether there is a difference in HRG concentration between patients who incurred an aSAH and those who did not, and between patients who developed a vasospasm and those who did not. We also investigated the usefulness of HRG as an early predictor of CVS. A second goal was to determine whether HRG interacts with other potential biomarkers. We measured and evaluated interleukin (IL)-6, an inflammatory marker, fibrinopeptide A (FpA), a coagulation marker, and 8-hydroxy-2'-deoxyguanosine (8OHdG), an oxidative stress marker.

Materials and Methods

Ethical approval

For this type of retrospective study, formal consent of the patients was not required. All

applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and all of the procedures in the present animal experiments were in accordance with the ethical standards of our institution.

Background characteristics

We retrospectively analyzed the cases of patients with aSAH and controls. They were of comparable age and gender. We classified the aSAH patients into spasm and non-spasm groups. The aSAH patients were evaluated both at their admission with the Hunt and Kosnik grading (H&K grading) and the World Federation of Neurological Surgeons SAH scale (WFNS scale), as well as at discharge using the modified Rankin Scale (mRS).

Aneurysmal neck clipping or coil embolization to prevent re-rupture was performed in the patients who were of treatment adaptation. In addition, in the spasm group, we evaluated whether the patients developed a symptomatic spasm and/or DCI. All assigned patients received medical treatment in our hospital's intensive care unit for ≥ 2 weeks. Standard prophylactic treatments were performed to prevent vasospasm or DCI. Each CVS was detected by digital subtraction angiography (DSA) or magnetic resonance angiography (MRA) 7–9 days after its onset. DCI was confirmed by

computer tomography (CT) or magnetic resonance imaging (MRI) with proper timing.

When it was thought necessary for CSF drainage, a spinal tube was inserted appropriately. Each patient's sample study was gathered at the same time that the CSF was submitted for a clinical examination.

We enrolled the total of 40 aSAH patients who were admitted to Kagawa University Hospital from September 2014 to September 2016. CSF and serum samples were gathered several times per patient between day 0 and day 11. We allocated these samples to three phases (T1–T3). We defined T1 as the period within 48 hr following the patient's SAH. The T2 phase was after T1 and before the examinations for spasm. T3 was the phase after the spasm check. Thus, T1 reflects the period immediately after spasm onset, T2 the early or pre-spasm phase, and T3 the severe spasm phase.

We selected the cases of the patients in whom CSF samples could be gathered during T1 and T2 with or without T3, and we investigated several biomarkers including HRG. Serum HRG concentrations were calculated in some of the enrolled patients who had stored samples. We excluded the cases in which CSF samples were not obtained in the appropriate phase, the cases in which the treatment for preventing re-rupture was not performed, and the cases of patients who died during their follow-up or suffered severe meningitis. The sample collection and retrospective analysis were approved by the

Ethics Committee of Kagawa University.

We also investigated relationship the between the value of T1 and the hematoma volume. The semiquantitative classification of hematoma is as follows. Each of 10 basal cisterns and fissures were graded separately on a semiquantitative scale according to the amount of extravasated blood, from 0 to 3. The total amount of subarachnoid blood (sum score) was calculated by adding the 10 scores, and the possible sum thus ranged from 0 to 30. There was a dose-dependent relationship between the T1 value and semiquantitative classification of the hematomas.

CVS detection

At our facility, all aSAH patients undergo preoperative DSA. Thus, when follow-up DSA is performed to check for a spasm, the images obtained before and after treatment can be compared. We classified the patients into a non-spasm group and a spasm group based on their DSA findings. We further divided the spasm group into the subgroup of patients who had experienced an angiographic spasm (i.e., a CVS around the circle of Willis or a peripheral artery, without neurological defect, including patients who had slight stenosis compared with preoperative findings) and the subgroup of patients who had experienced a symptomatic spasm (i.e., a CVS with a neurological defect such as

consciousness disorder, paralysis, and dysarthria).

Handling of CSF and serum samples

Within 15 min of collection, the CSF samples were centrifuged at 1000 g for 15 min, and the supernatants were collected and stored at -80°C . Before each assay was performed, high-molecular-weight proteins were removed by filtration (COSMOSIL, Kyoto, Japan). Serum samples were first left to stand for 30 min and then centrifuged at 300 g for 10 min. We did not use a reservoir for serum samples. The samples were stored as for CSF.

Concentrations of HRG, IL-6, FpA, and 8OHdG

We measured the targeted proteins and cytokines in the patients' CSF and serum with commercial quantitative sandwich enzyme-linked immunosorbent (ELISA) assay kits. HRG was measured by a kit from CUSABIO/CusAb (College Park, MD, USA), IL-6 was measured by a kit from (R&D Systems, Minneapolis, MN), FpA measured by a kit from CUSABIO/CusAb, and 8OHdG measured by a kit from JaICA (Shizuoka, Japan).

Standards were prepared and the appropriate volume of samples or standards were added to 96-well polystyrene microtiter plates pre-coated with a monoclonal

antibody to HRG, IL-6, FpA, or 8OHdG. The plates were incubated for the time recommended by the ELISA kit's manufacturer. Each well was then aspirated, and the plates were washed with the buffered surfactant provided. An enzyme-linked polyclonal antibody against each element was then added, and the plates were incubated and washed. Substrate solution was added to each well, and the optical density was read at a wavelength of 450 nm.

Rat preparation and the incubation of blood/CSF

To investigate the time course of the HRG concentration in CSF, we made a rat blood/CSF model. Three 8-week-old Sprague-Dawley rats weighing approx. 250 g had a femoral artery exposed and a catheter inserted in the aorta to obtain 0.5 ml of arterial blood. These aliquots were mixed with 9.5 ml of artificial CSF (Funakoshi, Tokyo) and placed in an incubator at 37°C and aged under sterile conditions in the dark for ≤ 240 hr. At 3, 48, 96, 144, 192 and 240 hr, 1.0 ml of blood/CSF was harvested. Each sample was centrifuged at 1000 g for 15 min, and the supernatant collected and stored at -80°C . The HRG concentration was then measured with an ELISA kit from CUSABIO/CusAb.

ROC analysis

We obtained non-parametric receiver operating characteristic (ROC) curves and the area under the curve (AUC) to quantify how well HRG discriminated between the spasm and non-spasm groups. The AUC can be considered a simple measure of the probability that a randomly selected case would have a higher biomarker value than a control.

Statistical analysis

Data are presented as the mean \pm standard deviation (SD). The statistical analyses were done with StatMate V software (ATMS, Tokyo, Japan). We used Welch's t-test or an analysis of variance (ANOVA) which is supported by LSD (least significant difference) to analyze the data. Statistical significance was defined as $p < 0.05$.

To clarify the precise utility of HRG, we calculated the sensitivity, specificity, and AUC by obtaining the (ROC) curves with the use of JMP 13 software (SAS, Cary, NC).

Results

Table 1 summarizes the background characteristics of the patients. Of the 40 patients who suffered an aSAH during the target period, 26 were excluded. A spinal tube was not inserted in 15 patients. CSF was not collected in 10 patients with the proper timing. One patient suffered from bacterial meningitis because of the spinal tube during observation.

No patient was prevented from undergoing treatment for re-rupture prevention, and no patient died during their observation period.

The 14 enrolled patients were eight males and six females aged 53.4 ± 15.4 years (range 32–84 years). Of the aSAH group, seven patients incurred a CVS. Of these, three patients' condition developed to an angiographical CVS and four to a symptomatic CVS. Of the symptomatic CVS group, one patient received an intra-arterial injection of fasudil hydrochloride hydrate, and three underwent a balloon angioplasty. However, notwithstanding this aggressive treatment, one patient developed DCI.

The non-spasm patients and the spasm patient did not differ significantly by age, gender, SAH grade (H&K grading, WFNS grading), surgical procedure (coil/clip rate) or mRS at discharge. The sampling interval for CSF allocated to the T1, T2 and T3 phases also did not differ between these two patient groups. Among the patients who suffered an aSAH, 10 (four spasm patients, six non-spasm patients) had their serum HRG concentrations measured in addition to their CSF HRG levels.

Seven patients (five males, two females) were enrolled as the control group. Five had idiopathic normal pressure hydrocephalus (iNPH) and two had a benign brain tumor. The controls were older than the aSAH patients at 66.9 ± 20.6 years.

Figure 1 compares the CSF HRG concentrations in the aSAH groups and controls

in the initial T1 time period. Significantly higher HRG levels were revealed in the aSAH group ($324.9 \pm 300.5 \mu\text{g/ml}$) compared to the control group ($54.3 \pm 19.3 \mu\text{g/ml}$; $p < 0.05$), and the spasm group's HRG levels were higher than those of the non-spasm group ($133.4 \pm 68.5 \mu\text{g/ml}$), but not significantly so.

We also investigated whether there is a relationship between the T1 value and hematoma volume. Each of 10 basal cisterns and fissures were graded separately on a semiquantitative scale, according to the amount of extravasated blood from 0 to 3. The total amount of subarachnoid blood (sum score) was calculated by adding the 10 scores, providing a possible total score from 0 to 30 [13]. The results indicated a positive correlation between the T1 value and hematoma volume (Fig. 2).

Figure 3A,B shows the time courses of the patients' CSF and serum HRG concentrations during phases T1–T3 in the aSAH group. The standard value of serum concentration was set based on a previous report [6], and we calculated the standard value of the CSF concentration based on the average value of control group. In both the aSAH and control groups, the serum HRG concentrations were almost the same as the standard, and the amount of change with time was small. In contrast, the CSF HRG concentrations were extremely high in both aSAH groups compared to the average of the control group.

We use the CSF data to calculate the change in the HRG level with time, with T1 set as zero (Fig. 3C). In the non-spasm group, the T2 and T3 values were higher than the T1 values (changes from T1 at T2: 37.7 ± 86.1 $\mu\text{g/ml}$, at T3: 87.3 ± 184.2 $\mu\text{g/ml}$). In contrast, the T2 and T3 values were lower in the spasm group compared to the T1 values (changes from T1 at T2: -107.2 ± 113.2 $\mu\text{g/ml}$, T3: -69.6 ± 136.2 $\mu\text{g/ml}$). There were significant differences among the groups ($p < 0.01$). However, there was no difference between the angiographic spasm and symptomatic spasm groups (data not shown). The detailed data for each patient are shown in Figure 4.

Figure 5 shows the time courses of the IL-6, FPA, and 8OHdG concentrations in CSF. As with HRG, these concentrations were measured at the T1, T2 and T3 time periods. Regarding IL-6, in both groups, the CSF concentrations at T2 and T3 were higher than at T1 (spasm group, T1: 2840 ± 1884 pg/ml , T2: 3028 ± 1825 pg/ml , T3: 4089 ± 1274 pg/ml ; non-spasm group, T1: 2593 ± 1766 pg/ml , T2: 3662 ± 1768 pg/ml , T3: 2666 ± 1626 pg/ml). The IL-6 concentration gradually increased and reached a maximum at T3 in the spasm group.

In contrast, in the non-spasm group, the IL-6 concentration decreased after it reached a maximum at T2. During T3, the IL-6 concentration was higher in the spasm group than the non-spasm group, but this did not quite reach significance ($p = 0.055$, Fig.

5A). The concentrations of FpA were highest at T1 (spasm group: 1.47 ± 1.33 ng/ml, non-spasm group: 0.29 ± 0.57 ng/ml) and then rapidly decreased. All of the T2 and T3 samples in both groups were below measurement sensitivity (<0.039 ng/ml). The T1 concentration of FpA was higher in the spasm group, but this did not quite reach significance ($p=0.053$, Fig. 5B). As for 8OHdG, the T1 concentrations were the highest (spasm group: 10.3 ± 10.2 μ g/ml, non-spasm group: 7.0 ± 2.1 μ g/ml), and the concentrations then gradually decreased (spasm group, T2: 3.9 ± 0.1 ng/ml, T3: 3.8 ± 0.7 ng/ml; non-spasm group, T2: 4.0 ± 2.2 ng/ml, T3: 3.2 ± 0.5 ng/ml). However, there were no significant differences between the spasm and non-spasm groups (Fig. 5C).

Figure 6 shows the time course of the HRG concentrations in the rat blood/CSF incubation model. The concentration was measured at 3, 48, 96, 144, 192, and 240 hr after blood/CSF incubation. The HRG concentration peaked at 48 hr and decreased thereafter. However, all of the values after 48 hr were higher than the 3 hr value. This HRG time course was similar to that found for CSF in the samples from the non-spasm patients.

Figure 7 shows the ROC curves regarding the prediction of CVS at arbitrarily chosen HRG cutoff values. We used the amount of change in the T2 values with T1 set as zero. The cutoff value of -36.6 μ g/ml for HRG showed 85.7% sensitivity and 71.4%

specificity for the prediction of a CVS during the T2 phase. The AUC of this ROC was 85.7%.

Discussion

We consider the following hypothesis regarding the etiology of CVS. As the first step, a strong coagulation disorder occurs, which leads to a decrease in HRG that is caused by the consumption. The decrease in HRG also induces inflammation. A series of cascades causes a vascular endothelial injury, resulting in a CVS.

HRG is a 75-kDa plasma glycoprotein produced in and secreted from the liver [14]. It is thought to be involved mainly in the regulation of coagulation and fibrinolysis. HRG inhibits the contact pathway activation of plasma *in vitro*, presumably because the histidine-rich region of HRG binds to negatively charged surfaces and prevents the auto-activation of FXII (coagulation factor XII) [3,4]. In support of this concept, HRG-deficient mice exhibit shortened clotting times [5]. On the other hand, it was also reported that HRG participates in the immune response [6] and angiogenesis [7–10]. Interestingly, Wake et al. have clearly shown a novel and important role of HRG in controlling neutrophil shape, adhesiveness, and basal reactive oxygen species (ROS) producing activity [8]. The neutrophil shape maintained by HRG should be suitable for

the passage of neutrophils through capillary vessels, sustaining rheological stability, and preventing the unnecessary activation of endothelial cells that can lead to vascular injury. Thus, decreased HRG may lead to vascular injury and the production of inflammatory cytokines such as IL-6.

Plasma HRG concentrations in healthy humans are reported to be constant at approx. 1 mM [6]. However, the CSF concentrations of HRG have never been reported. In the present study, we considered the average value of the control group as a standard value, approx. 0.7 μ M. The HRG concentrations in the CSF of our aSAH patients were extremely high for the entire study period compared to the standard value. In contrast, the drastic changes observed in the CSF were not seen in the patients' serum. It is likely that we were not able to detect changes in the serum because pathological changes in aSAH occur locally in the subarachnoid space. Thus, for further examinations of the importance of HRG, CSF concentrations are more useful than those in serum.

The relationship between biomarkers and CVS

Understanding the pathophysiology of CVS and early risk assessment are still major challenges in SAH. A CVS occurs due to the continuous contraction of one or several areas of smooth muscle in the intracranial vasculature, or vascular injury, which leads to

changes in the luminal morphology and intracranial vascular stenosis or spasm [15].

Various biomarkers (e.g., inflammatory, oxidative stress, and coagulation system markers) have been investigated in SAH, and we examined such biomarkers to judge their usefulness compared with HRG. We selected IL-6 as an inflammatory marker, FpA as a coagulation system marker, and 8OHdG as an oxidative stress marker.

IL-6 is a pleiotropic inflammatory cytokine of low molecular weight [16]. After an aSAH, the CSF concentration of IL-6 peaks at 6–7 days and declines by approx. 10 days [17]. These findings suggested that a severe inflammatory response occurs in the brain. Unfortunately, inflammation in CSF can induce a persistent and severe CVS [18]. Emerging evidence indicates that high concentrations of IL-6 in the plasma or in the CSF are associated with a significant high risk of CVS, DCI, and poor outcome after aSAH [19–24]. It was also indicated that IL-6 not only reflects a current vasospasm but may also be an early biomarker for predicting vasospasm after SAH [25,26]. Our present results support this idea. In our spasm group, the maximum IL-6 concentrations were confirmed at the T3 phase, suggesting persistent inflammation in CVS cases.

Fibrinopeptide A is cleaved from fibrinogen by thrombin during coagulation [27–29]. When an aSAH occurs, the balance between the coagulation and fibrinolytic systems in the CSF is altered. It is thus understandable that various coagulation factors

were reported to be more strongly activated within 3 days after an aSAH [30]. Kasuya et al. observed extremely high FpA levels in the CSF of patients during the immediate post-aSAH phase compared to healthy humans [31]. Higher concentrations of FpA have been reported in DCI compared to non-DCI patients [32–34]. These changes occurred within 48 hr of aSAH onset. Subsequently, the FpA concentration rapidly decreased in both groups [34]. In the present study, the FpA concentrations during the T1 period were higher in the spasm group, indicating that the coagulopathy in the CSF was more marked in this group. As in previous reports, the FpA concentration decreased rapidly. Thus, the changes in coagulopathy in the CSF during the immediate phase may be related to CVS indirectly or directly.

8-Hydroxy-2'-deoxyguanosine, derived from a hydroxyl radical attack of deoxyguanosine residues, is the most representative product of DNA oxidative modification [35,36]. Oxidative stress plays a significant role in the processes of acute brain damage after SAH as well as cerebral vasospasm [37]. It was shown that 8OHdG peaks at day 2 after SAH and then gradually decreases and correlates to CVS and following DCI [38,39]. We observed no clear difference in the 8OHdG concentration between our non-spasm and spasm groups. However, the finding that the peak CSF 8OHdG concentration occurred during the immediate (T1) period suggests that

oxidative stress occurs in the immediate phase after an aSAH.

The relationship between HRG and CVS

This is, to the best of our knowledge, the first publication studying HRG concentrations in the CSF during the acute phase of aSAH. Our extensive literature search revealed only one article reporting an influence of HRG on cerebral arteries; those researchers reported that HRG causes a dose-dependent dilation of porcine cerebral arterioles [40]. A decrease in HRG may thus result in cerebral vessel contraction. We observed that the CSF HRG concentration in the T1 period correlated with the hematoma volume.

Considering that the HRG concentrations in T1 were higher in the spasm group, we speculate that a hematoma in the subarachnoid space affects the development of a CVS, as other researchers have suggested. There were also more remarkable differences among the groups during the T2 phase; the HRG concentration was decreased in the spasm group whereas it increased in the non-spasm group. During T3, a similar tendency was observed.

In the blood/CSF rat model, the HRG concentration followed a time course that was similar to that of the CSF HRG concentration in the human non-spasm group. The increased HRG concentration may be due to the release of free HRG found in plasma as

well as the release of HRG stored in the α -granules of platelets [41,42]. We thus suggest that the consecutive release of HRG from an aging clot caused the local increases in HRG in the human non-spasm group and in the blood/CSF model. Samantha et al. measured the time course of the HRG release from aging clots [40], and they found that the HRG concentration was significantly elevated during 24–72 hr of culture, which is in agreement with our results. The decrease in CSF HRG with time in our group of spasm patients may be related to pathological changes triggered in that group.

Our evaluation of biomarkers in CSF related to CVS in aSAH patients demonstrated that increased FpA at T1, decreasing levels of HRG at T2, and increased IL-6 levels at T3 occurred in the spasm group. In addition, although no clear differences between the spasm and non-spasm groups were observed, increased 8OHdG in the immediate phase was confirmed in the spasm group.

A proposed pathological mechanism underlying CVS based on these results is as follows. In the CSF of aSAH patients who develop a CVS, first, a strong coagulation disorder occurs, and then HRG decreases due to metabolism/clearance and induces inflammatory cytokines. These cytokines cause vascular endothelial injury, resulting in a CVS. This proposed pathway suggests that HRG may form a part of a pathologic pathway in CVS. To test these hypotheses, additional experiments are necessary.

The usefulness of HRG as an early predictor of spasm

We examined the usefulness of the CSF level of HRG for predicting a spasm, in a comparison with three other biomarkers. Our results suggested but did not prove a relationship between the FpA and IL-6 concentrations with CVS. On the other hand, we did not detect a difference in the CSF 8OHdG levels between the spasm and non-spasm groups. Various biomarkers—including those in the present study—have been examined, but no useful early predictor for CVS has been established. In our study, the CSF's level of HRG decreased at the T2 phase in the spasm group, with a significant difference between the spasm and non-spasm aSAH groups. We examined whether HRG might become an early predictor of CVS. The usefulness of HRG during the T2 period was statistically excellent. The T2 period is before DSA or MRA is performed to check for a spasm in angiographic spasm patients, and before suffering from neurological deficits in the symptomatic spasm group. HRG may thus become a biomarker that can detect changes in the early or preliminary phase of a CVS, and its diagnostic usefulness may be extremely high.

Regarding possible limitations of the present study, the number of samples and the collection days were not uniform, as the CSF samples of aSAH patients were

examined retrospectively. The pathophysiology of aSAH can vary by individual, and the onset day of a neurological deficit with a symptomatic spasm can also differ. Therefore, we do not know whether we can allocate such diversity into three time periods uniformly. In addition, in the patients with a mild SAH, the hematoma was washed out around onset day 10 and their HRG markedly decreased, causing unevenness in the data. A prospective study is needed to resolve these issues. In such a study, we will increase the number of samples per patient and sample at fixed intervals.

In conclusion, we investigated the time course of HRG in the cerebrospinal fluid after aSAH patients for the detection of a cerebral vasospasm. The results suggest that the CSF concentration of HRG has the possibility to become an early predictor of CVS. In addition, the interactions of HRG with IL-6, FpA and 8OHdG may form part of the pathology of CVS. A future prospective study will investigate the relationship between the pathology of CVS and HRG.

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COI disclosures

None.

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Table 1. Patient demographics

N	control group	aSAH group		P value
	7	14		
		spasm	nospasm	
	7	7		
age	66.9±20.6	57.3±16.2	56.4±15.0	NS
gender (male: female)	5:2	4:3	4:3	NS
SAH grading				
H & K grade				NS
I		0	0	
II		4	3	
III		1	1	
IV		1	2	
V		1	1	
WFNS grade				NS
I		2	1	
II		3	2	
III		0	0	
IV		0	2	
V		2	2	
T1 (day)		0.57±0.53	0.50±0.55	NS
T2 (days)		4.71±1.38	5.17±1.47	NS
T3 (days)		8.71±1.98	8.67±1.21	NS
surgical procedure (coil: clipping)		5:2	6:1	NS
symptomatic spasm		4		
DCI		1		
mRS		2.57±1.6	2.00±1.5	NS

Figure Legends

Fig. 1. The CSF HRG concentrations during the T1 period in the control, non-spasm, and spasm aSAH patients. The HRG levels were significantly higher in the spasm aSAH patients compared to the controls and higher in the spasm group than the non-spasm group.

Fig. 2. The relationship between the T1 value and the hematoma volume. The results indicate a positive correlation ($p < 0.01$).

Fig. 3. The CSF HRG concentrations in the T1–T3 phases in the non-spasm (A) and spasm (B) aSAH groups. *Black dotted line:* The average CSF concentration in the control patients. *Gray dotted line:* The mean serum HRG concentration. In both the non-spasm and spasm groups, the serum HRG concentrations were almost the same as those of the control patients, and the amount of change with time was small. In contrast, the CSF HRG concentrations were much higher in both aSAH groups compared to the controls, and this difference persisted with time. **C:** The changes in the CSF HRG concentration at T2 and T3 compared to T1 (set as 0). In the non-spasm aSAH group,

the T2 and T3 values were higher than the T1 values, whereas they were significantly lower in the spasm group ($p<0.01$).

Fig. 4. Each patient's HRG concentration changes. The HRG concentration tended to rise in the non-spasm group and tended to decline in the spasm group.

Fig. 5. The time courses of potential biomarkers in the CSF in the spasm and non-spasm aSAH patients. **A:** The IL-6 concentrations during phases T2 and T3 were higher than those in T1 in both groups. In the spasm group, the concentration reached a maximum in T3. In the non-spasm group, after the concentration reached a maximum during T2, the concentration decreased. There were no significant differences between the spasm and no-spasm groups at any time point, although at T3 the spasm group was nearly significantly higher ($p=0.055$). **B:** The CSF FpA concentration in both groups was highest in T1, and rapidly decreased. All T2 and T3 samples in both groups were below the measurement sensitivity ($p<0.039$ ng/ml). In T1, the CSF FpA concentration was higher in the spasm group, but this was not quite significant ($p=0.053$). **C:** In both the spasm and non-spasm groups, the CSF 8OHdG concentrations were highest in T1 and

gradually decreased. There were no significant differences between the two groups at any time point.

Fig. 6. The HRG concentration time course after blood/CSF incubation. The concentration peaked at 48 hr and decreased thereafter. However, all of the samples after the 48-hr value were still higher than the 3-hr value.

Fig. 7. ROC curves regarding the prediction of CVS.

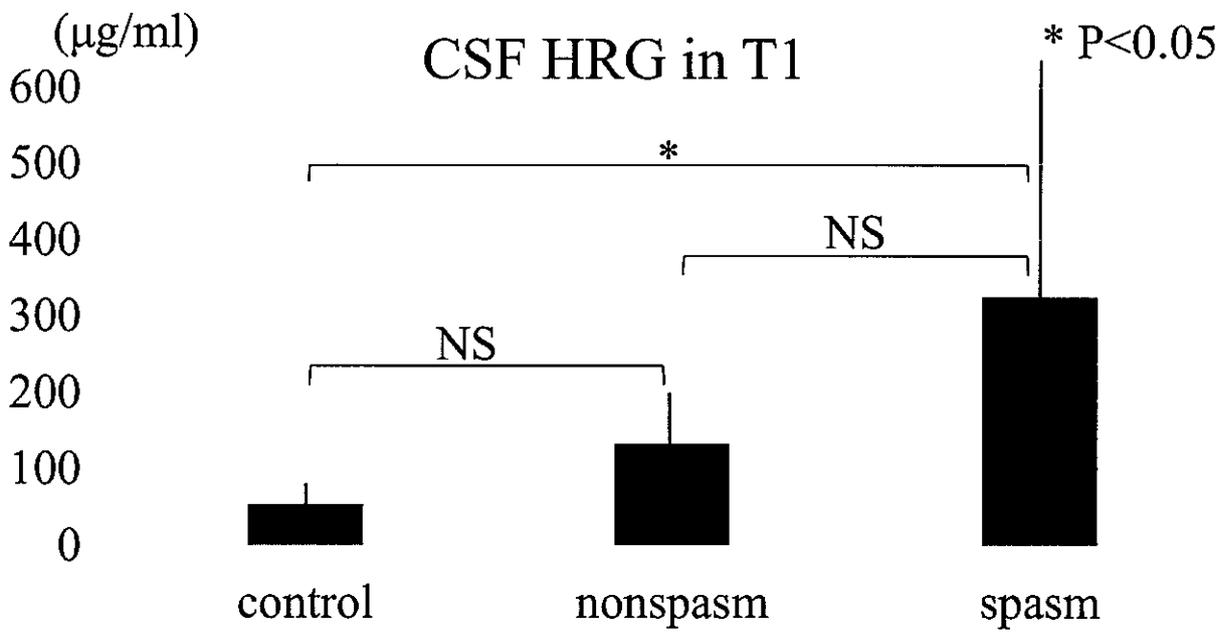


Figure 1

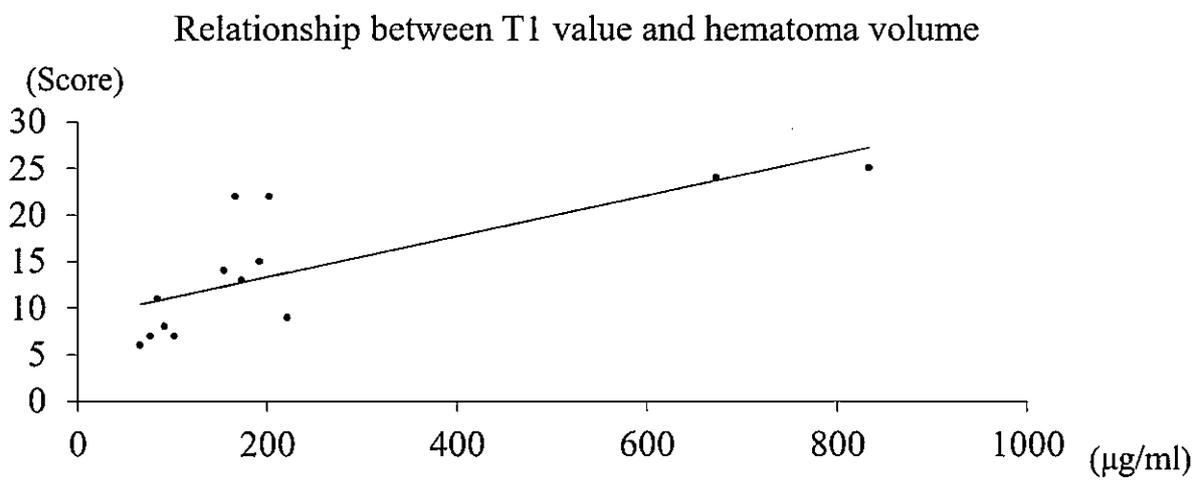


Figure 2

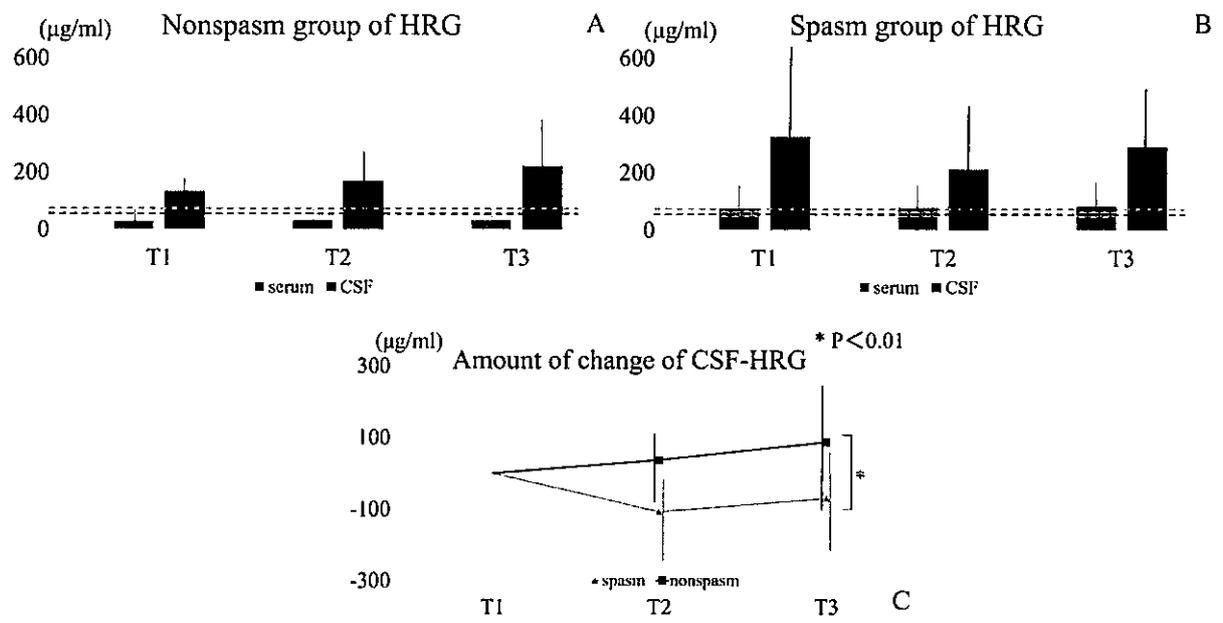


Figure 3

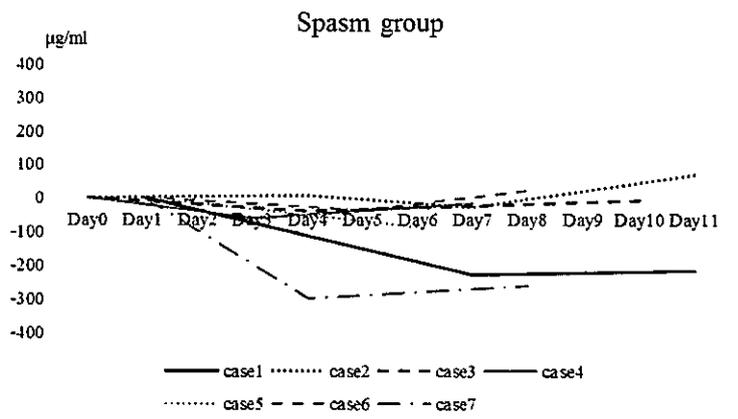
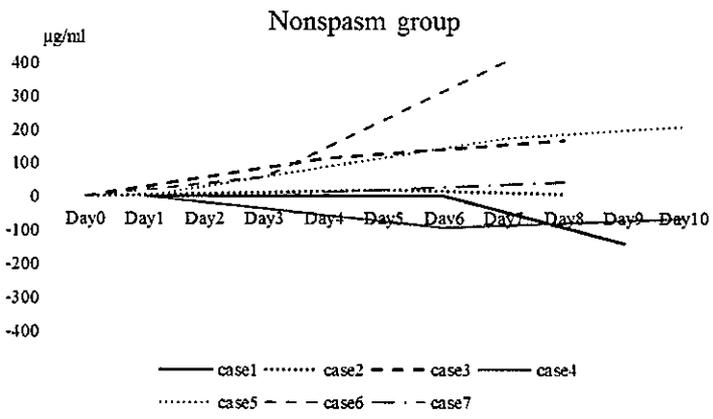


Figure 4

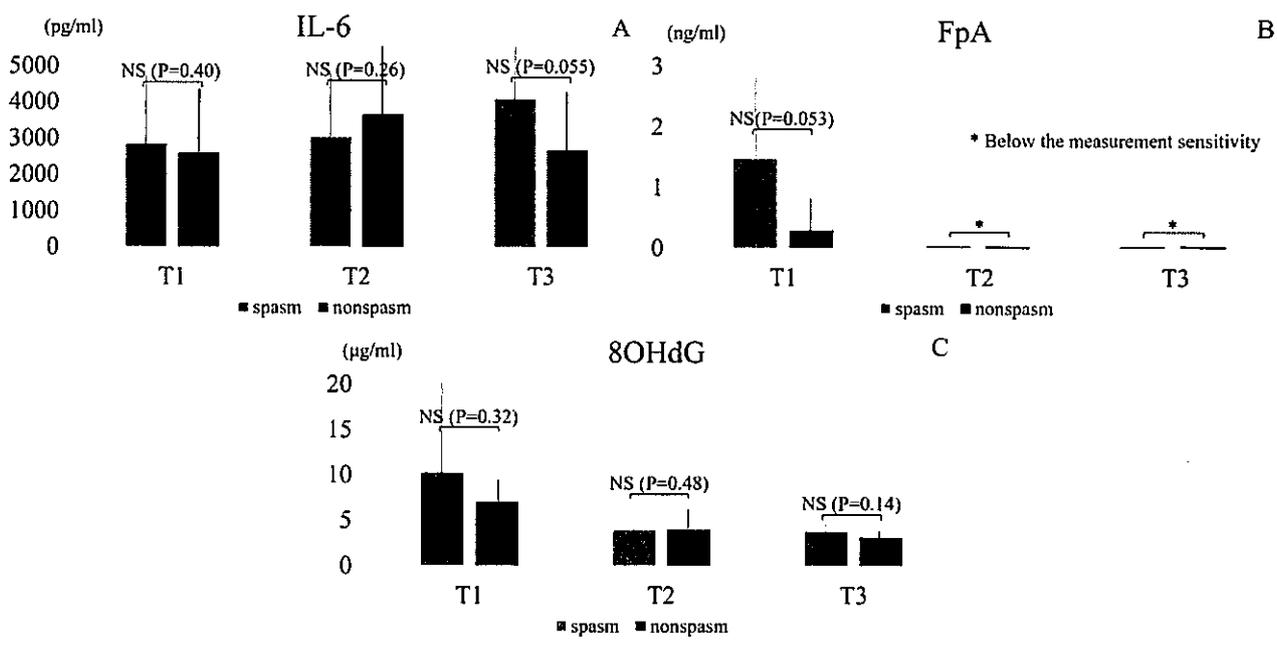


Figure 5

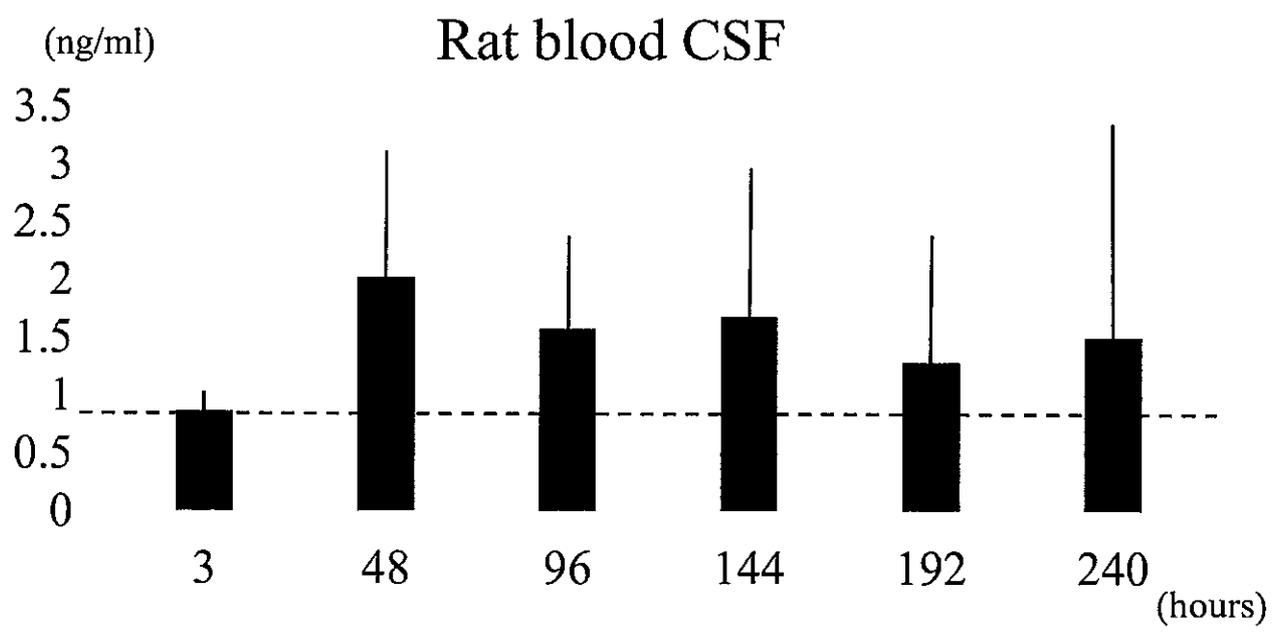


Figure 6

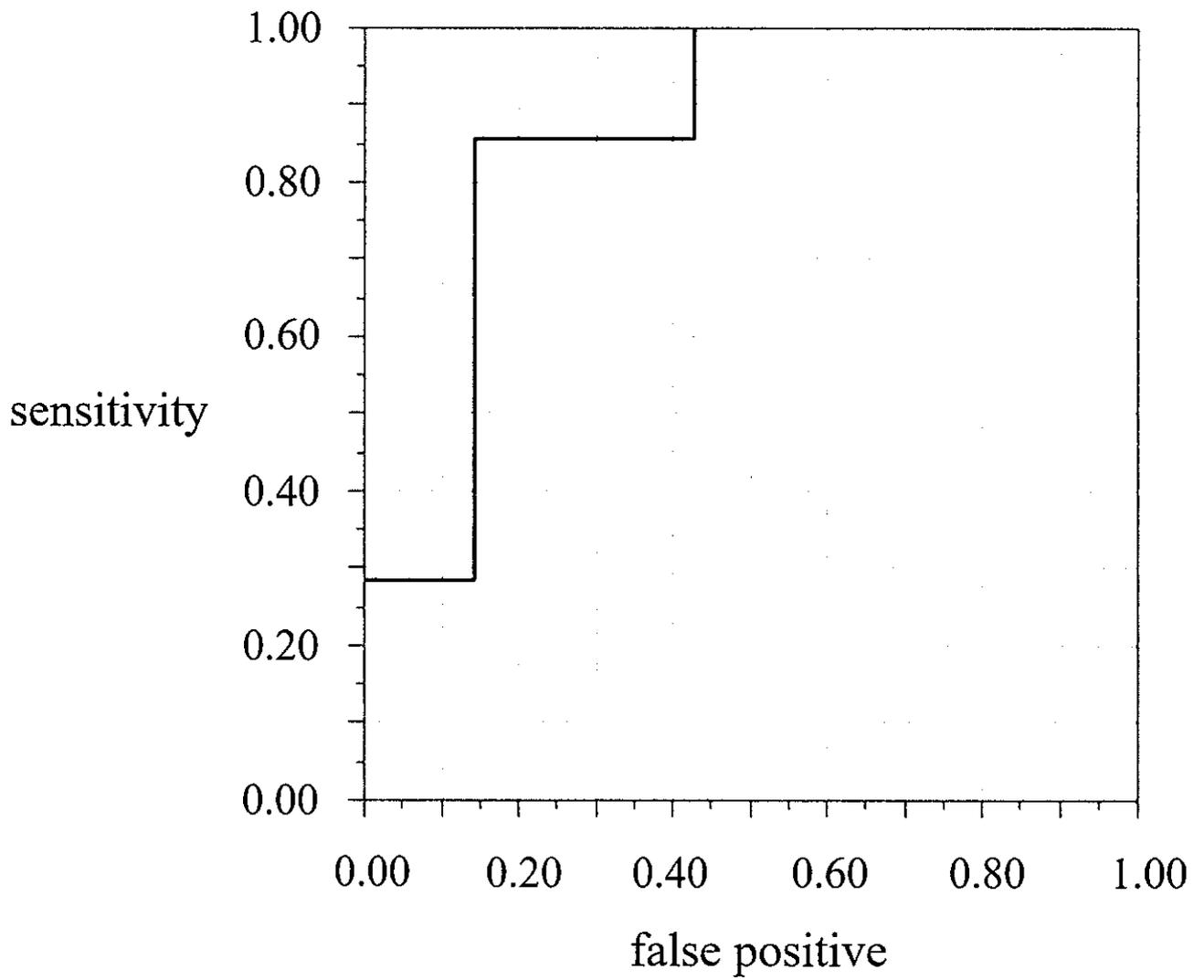


Figure 7