Intracerebral hemorrhage (ICH) accounts for ≈10% to 15% of strokes in Western countries and up to 20% to 30% in Asian countries. ICH is associated with higher mortality and worse clinical outcome than ischemic stroke, and no effective therapy is available. Subsequent to the initial physical trauma and mass effect of the bleeding, secondary brain injury, such as perihematomal edema (PHE) including vasogenic (extracellular) and cytotoxic (intracellular) edema, develops. The impact of PHE on clinical outcome is still under debate.

Matrix metalloproteinase (MMP)-3 and MMP-9, as well as growth factors (GFs) such as vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1), are expressed in abnormally high concentrations in brain and peripheral blood in ICH patients. In ICH animal models, elevated MMP-9 and MMP-3 contribute to blood–brain barrier (BBB) disruption and neuronal cell death.

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Method — Fifty-nine patients with spontaneous ICH admitted within 24 hours of symptom onset were prospectively investigated. Noncontrast CT was performed on admission for diagnosis of ICH and quantification of initial hematoma volume. MRI was performed on day 3 to evaluate perihematomal edema. Concentrations of MMP-3, MMP-9, as well as vascular endothelial growth factor and angiopoietin-1 on admission were determined by enzyme-linked immunosorbent assays. Clinical outcome was assessed by modified Rankin Scale at 90 days.

Results — Increased MMP-3 levels were independently associated with perihematomal edema volume (P<0.05). Cytotoxic edema surrounding the hematoma was seen in 36 (61%) cases on 3-day MRI. Cytotoxic edema did not correlate with the level of any of the biomarkers studied. Levels of MMP-3 ≥12.4 ng/mL and MMP-9 ≥192.4 ng/mL but not vascular endothelial growth factor and angiopoietin-1 predicted poor clinical outcome at 90 days (modified Rankin Scale >3) independent of stroke severity and hematoma volume at baseline (odds ratio, 25.3, P=0.035; odds ratio, 68.9, P=0.023; respectively).

Conclusions — MMPs 3 and 9 seem to be significantly involved in secondary brain injury and outcome after primary ICH in humans, and thus should be further evaluated as targets for therapeutic strategies in this devastating disorder. (Stroke. 2013;44:658-663.)
Methods

Study Population
All patients with spontaneous ICH admitted to the Neurological Department of Beijing Tiantan Hospital from January 2011 to December 2011 were screened for this study. Inclusion criteria were time from symptom onset to admission <24 hours, age <80 years, and absence of coma. Exclusion criteria were secondary ICH (hemorrhage resulting from aneurysm, vascular malformation, hemorrhagic infarction, tumor, or impaired coagulation), history of acute or chronic infection, malignant diseases and immunosuppressive treatment, contraindication for MRI, undergoing a surgical procedure, or refusal of participation. A total of 59 patients were prospectively included after informed consent from patients or their relatives. The study has been approved by the local ethics committee.

Clinical data of patients were collected on admission. The variables included sex, age, body mass index, alcohol and tobacco use, a detailed history of vascular risk factors and concomitant medications, body temperature, systolic and diastolic blood pressure, and laboratory tests. Stroke severity was evaluated by Glasgow Coma Scale and National Institutes of Health Stroke Scale at admission. Poor clinical outcome was defined as modified Rankin Scale >3 assessed at 90-day follow-up.

Radiological Protocol
Noncontrast cerebral computed tomography (NCCT) scans were performed on admission, according to the protocol of the neuroradiological department with an image matrix of 512×512, 4.5-mm-wide slices for posterior fossa, and 9-mm-wide slices for medium and anterior fossae. Cerebral MRI was performed by 3.0 Tesla scanners (Trio-Tim, Siemens, Erlangen, Germany) on 3±1 days. MRI included the following sequences: conventional T1-weighted and T2-weighted images, gradient-recalled echo imaging (T2*), diffusion-weighted images (DWI) using 2 levels of diffusion sensitization (b=0 and 1000 s/mm2), and apparent diffusion coefficient (ADC) maps created from DWI images by the image analysis system. Additionally, in 50 patients, a follow-up NCCT was done at 24 hours as a clinical routine.

Investigators who analyzed the images were blinded to clinical and biomarker information. Hematoma location (supratentorial deep location versus others, including lobar and infratentorial locations), and the presence of intraventricular hemorrhage (IVH) were recorded. Hematoma volume and total lesion area were calculated by the summation-of-area method on each slice multiplied by the interslice thickness on both NCCT and 3-day MRI. Hematoma expansion was defined as an increase of hematoma volume >12.5 mL from symptom onset to baseline NCCT as measured on 3-day T2-weighted images. Relative PHE volume was defined as absolute PHE volume divided by hematoma volume, yielding a dimensionless ratio variable. Areas of increased DWI-b1000-signal and reduced ADC value by >10% compared with the mirror region of interest were interpreted as cytotoxic edema (CE) and manually outlined,7 if located outside of the ICH on T2*- and DWI-b0-images. This was confirmed by a 3D-multiplanar localization function of the image analysis software (Figure 1).

Immuoassay Methods
For the measurement of markers, blood samples were drawn using EDTA and serum tubes from each patient on admission. Plasma was immediately separated by centrifugation at 3000 rpm for 15 minutes and stored at −80°C. The levels of MMP-3, MMP-9, VEGF, and Ang-1 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer instructions (R&D Systems). MMP-9 was measured in EDTA plasma,23,24 MMP-3, VEGF, and Ang-1 in serum.

Statistical Analysis
Statistical analysis was performed using the SPSS statistical package Version 11.5. Categorical variables are shown as numbers and percentages. Continuous variables are presented as mean±SD, or median values [interquartile range] as appropriate. Tests performed were the χ2 or Fisher exact tests for categorical variables, and the Student t test or the Mann–Whitney U test for continuous variables as appropriate. Spearman correlation analysis was used to study correlations between continuous variables. Cut-off points of studied markers were determined using the receiver operating characteristic curve and Youden Index. Multivariable analysis using a multiple linear regression model was performed to assess the relationship between the molecular markers and the PHE volume at day 3. A stepwise logistic regression analysis was performed to determine factors that could be considered independent predictors of unfavorable clinical outcome at 3 months. Variables were retained in the logistic regression model for P≤0.10. A value of P<0.05 was considered significant.

Results

Baseline Clinical, Neuroimaging, and Laboratory Findings
Baseline variables are shown in Table 1 (n=59). Median time from symptom onset to baseline NCCT was 3.5 (1.6–7.5) hours. Twenty-four–hour follow-up CT was performed in 50 patients. The median hematoma volume on baseline and 24-hour follow-up CT was 9.9 (5.4–21.3) ml and 10.9 (5.6–25.8) ml, respectively (n=50). Hematoma expansion was found in 3 patients who had follow-up CT and in none of the

Figure 1. Example of edema surrounding the hematoma on 3-day MRI. Black and white arrows indicate hematoma and perihematomal edema (PHE) on the T2-weighted image, respectively. Region of interest of cytotoxic edema (CE) is outlined on the apparent diffusion coefficient (ADC) image.
remaining 9 patients who only underwent follow-up MRI. MMP-3, MMP-9, VEGF, and Ang-1 levels did not correlate with hematoma volume at baseline. No correlation between these molecular markers and the absolute increase of hematoma volume was found ($P>0.05$).

**Association of PHE Volume With Hematoma Volume and Molecular Markers**
Median time to follow-up MRI was 76 (69–88) hours. PHE was present in all patients on 3-day T2-weighted images. A high correlation was found between absolute PHE and hematoma volume at baseline and day 3 ($r=0.922$ and $r=0.959$, respectively; both $P<0.001$; Figure 2). Absolute PHE volume was positively correlated with MMP-3 ($r=0.311$; $P=0.017$), but not with MMP-9, VEGF and Ang-1. Hematoma volume on day 3 was positively correlated with MMP-3 ($r=0.311$; $P=0.017$) and negatively correlated with Ang-1 ($r=-0.281$; $P=0.031$), but did not correlate with MMP-9 nor with VEGF. The linear stepwise regression model revealed that MMP-3 was independently associated with absolute PHE volume ($\beta=0.370$; $P=0.004$), irrespective of age and gender. MMP-3 ($\beta=0.138$; $P=0.043$) remained independently associated with absolute PHE volume when baseline hematoma volume ($\beta=0.868$; $P<0.001$) was also considered. However, only hematoma volume on day 3 ($\beta=0.942$; $P<0.001$) remained as an independent predictor of PHE volume when it was included in the multivariate model instead of hematoma volume on baseline.

**Table 1. Baseline Clinical, Radiological, and Laboratory Characteristics of All Patients and Patients Grouped by 90-Day Outcome**

<table>
<thead>
<tr>
<th></th>
<th>All (n=59)</th>
<th>Favorable Outcome (n=50)</th>
<th>Unfavorable Outcome (n=9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male sex</td>
<td>41 (69.5)</td>
<td>35 (70.0)</td>
<td>6 (66.7)</td>
<td>1.000</td>
</tr>
<tr>
<td>Age, y</td>
<td>56±11</td>
<td>56±11</td>
<td>60±14</td>
<td>0.338</td>
</tr>
<tr>
<td>BMI</td>
<td>25.3±3.5</td>
<td>25.4±3.5</td>
<td>24.7±3.8</td>
<td>0.601</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>52 (88.1)</td>
<td>44 (88.0)</td>
<td>8 (88.9)</td>
<td>1.000</td>
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<tr>
<td>History of diabetes mellitus</td>
<td>11 (18.6)</td>
<td>9 (18.0)</td>
<td>2 (22.2)</td>
<td>0.670</td>
</tr>
<tr>
<td>History of stroke</td>
<td>13 (22.0)</td>
<td>12 (24.0)</td>
<td>1 (11.1)</td>
<td>0.673</td>
</tr>
<tr>
<td>Tobacco use</td>
<td>24 (40.7)</td>
<td>19 (38.0)</td>
<td>5 (55.6)</td>
<td>0.619</td>
</tr>
<tr>
<td>Alcohol use</td>
<td>26 (44.1)</td>
<td>22 (44.0)</td>
<td>4 (44.4)</td>
<td>1.000</td>
</tr>
<tr>
<td>Antihypertensive medication</td>
<td>26 (44.1)</td>
<td>21 (42.0)</td>
<td>5 (55.6)</td>
<td>0.697</td>
</tr>
<tr>
<td>Antiplatelet medication</td>
<td>8 (13.6)</td>
<td>6 (12.0)</td>
<td>2 (22.2)</td>
<td>0.494</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>168±25</td>
<td>165±24</td>
<td>180±25</td>
<td>0.108</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>97±15</td>
<td>98±15</td>
<td>91±15</td>
<td>0.163</td>
</tr>
<tr>
<td><strong>Imaging data on initial CT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematoma volume</td>
<td>10.0 [5.2–23.9]</td>
<td>9.5 [4.1–20.1]</td>
<td>25.2 [16.5–38.1]</td>
<td>0.003*</td>
</tr>
<tr>
<td>PHE volume</td>
<td>6.5 [3.0–11.9]</td>
<td>5.7 [2.5–11.7]</td>
<td>10.0 [6.7–22.1]</td>
<td>0.062</td>
</tr>
<tr>
<td>Deep location</td>
<td>49 (83.1)</td>
<td>40 (80.0)</td>
<td>9 (100)</td>
<td>0.322</td>
</tr>
<tr>
<td>Intraventricular hemorrhage extension</td>
<td>13 (22.0)</td>
<td>12 (24.0)</td>
<td>1 (11.1)</td>
<td>0.673</td>
</tr>
<tr>
<td><strong>Laboratory data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte, 10E9/L</td>
<td>9.0±2.7</td>
<td>8.9±2.7</td>
<td>9.9±3.1</td>
<td>0.313</td>
</tr>
<tr>
<td>Platelet, 10E9/L</td>
<td>220.7±47.3</td>
<td>218.5±49.1</td>
<td>232.9±34.9</td>
<td>0.406</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>7.0±2.1</td>
<td>7.2±2.2</td>
<td>6.0±0.8</td>
<td>0.144</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>64.4±18.4</td>
<td>64.2±19.6</td>
<td>65.6±10.2</td>
<td>0.844</td>
</tr>
<tr>
<td>INR</td>
<td>0.99±0.06</td>
<td>0.99±0.06</td>
<td>0.99±0.06</td>
<td>0.928</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>2.6±0.9</td>
<td>2.5±0.8</td>
<td>3.0±0.8</td>
<td>0.118</td>
</tr>
<tr>
<td>MMP-3, ng/mL</td>
<td>10.7 [7.5–21.0]</td>
<td>9.9 [6.9–18.7]</td>
<td>17.0 [10.3–28.1]</td>
<td>0.124</td>
</tr>
<tr>
<td>MMP-9, ng/mL</td>
<td>140.2 [102.4–217.0]</td>
<td>135.2 [100.4–192.2]</td>
<td>232.7 [97.7–306.8]</td>
<td>0.124</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>324.9 [186.7–516.2]</td>
<td>331.7 [197.2–523.2]</td>
<td>270.6 [164.6–404.9]</td>
<td>0.411</td>
</tr>
</tbody>
</table>

*P<0.05 was considered significant. Data are expressed as n (%), mean±SD, or median [IQR] as appropriate. Chi-square test was used for dichotomizing variables; the Student $t$ test or Mann–Whitney $U$ test was used for continuous variables.

Ang indicates angiopoietin; BMI, body mass index; GCS, Glasgow Coma Scale; INR, international normalized ratio; MMP, matrix metalloproteinase; NIHSS, National Institutes of Health Stroke Scale; PHE, perihematomal edema; and VEGF, vascular endothelial growth factor.
Figure 2. Relationship between baseline hematoma and 3-day perihematomal edema (PHE) volumes after intracerebral hemorrhage (ICH). Baseline hematoma volume vs 3-day PHE volume: r=0.922; P<0.001.

**Association of CE With Hematoma Volume and Molecular Markers**

Among the 59 included patients, CE was detected in 36 (61%) on 3-day MRI. Mean absolute and relative ADC value of CE were 527.5±83.4×10⁻⁶mm²/s and 0.665±0.116, respectively. Absolute and relative ADC value were negatively correlated with baseline hematoma volume (r=−0.337, P=0.044; r=−0.352, P=0.035; respectively). Patients with CE had a significantly larger hematoma volume at baseline and day 3 than those without (baseline: 14.7 [7.7–25.1] vs 6.9 [2.8–12.5] ml, P=0.011; day 3: 21.1 [10.0–41.3] vs 7.6 [2.9–17.5] ml, P=0.001; respectively; Figure 3). CE and ADC values did not correlate with MMP-3, MMP-9, VEGF, or Ang-1 levels. Radiological data and levels of measured markers grouped according to the status of CE are provided in Table 2.

**Association of PHE and Molecular Markers With Clinical Outcome**

At 90-day follow-up, 50 patients showed favorable outcome (modified Rankin Scale 0–3) and 9 unfavorable outcome (modified Rankin Scale 4–6). Clinical, radiological, and laboratory data according to outcome are presented in Table 1. Larger PHE volume on day 3 (P<0.001) but not on admission (P=0.062) was observed in those patients with unfavorable clinical outcome compared with those with favorable outcome. No association was found between outcome and presence or absence of CE. Receiver operating characteristic analysis was performed for concentrations of MMP-3, MMP-9, VEGF, and Ang-1. A possible cut-off point for the discrimination of dichotomized clinical outcome was calculated for MMP-3 as 12.4 ng/mL, MMP-9 as 192.4 ng/mL, VEGF as 317.6 pg/mL, and Ang-1 as 27.3 ng/mL. Using these cut-off points, univariate analysis found that MMP-3 ≥12.4 ng/mL and MMP-9≥192.4 ng/mL predicted unfavorable 90-day outcome (P=0.029 and P=0.018, respectively), whereas VEGF and Ang-1 did not (P=0.277 and P=0.252, respectively). Initial hematoma volume, Glasgow Coma Scale, and National Institutes of Health Stroke Scale score on admission were determined to be predictors of outcome as well. In addition to all the significant variables in univariate analysis, age, sex, and hematoma expansion were included in a multivariate logistic analysis to determine independent predictors for unfavorable outcome. As a result, MMP-3 ≥12.4 ng/mL (OR, 25.3; P=0.035), MMP-9 ≥192.4 ng/mL (OR, 68.9; P=0.023), and National Institutes of Health Stroke Scale score at baseline (OR, 1.7; P=0.005) were identified as independent predictors of unfavorable 90-day outcome.

**Discussion**

The main findings of the present study in acute patients with spontaneous ICH are: (1) the circulating levels of MMP-3 are correlated with absolute PHE volume; (2) increased levels of MMP-3 and MMP-9 at admission are independent predictors of poor clinical outcome; and (3) no association of the investigated markers (MMP-3, MMP-9, VEGF, and Ang-1) with the presence of CE was detected.

Our finding that baseline MMP-3 and MMP-9 levels predict poor clinical outcome, independently of stroke severity and baseline hematoma volume, can be explained pathophysiological. After ICH, secondary brain injuries, including BBB disruption, brain edema, and neuronal cell death, are induced by interaction of the coagulation cascade and thrombin production, erythrocyte lysis, and hemoglobin toxicity, together with inflammation. Pro-MMPs can be activated by plasmin, thrombin, hemoglobin products, and reactive oxygen species produced after ICH. Activated MMP-3 can activate MMP-9, which predominantly degrades the components of the extracellular matrix. In ICH animal models, several studies showed that elevated levels of MMP-9 lead to BBB breakdown and brain edema,12–15 It was recently demonstrated that MMP-9 and MMP-3 jointly contributed to blood-induced lesions and neuronal cell death.16,17 In previous clinical studies, MMP-9 levels significantly correlated with PHE volume in the first 24 hours after symptom onset.26,27 In our study, MMP-3 independently predicted PHE volume on day 3 after symptom onset. This phase of PHE is thought to result predominantly from BBB disruption and neuronal injury because of the interplay of hemoglobin toxicity and inflammation. Our study showed a relationship between 3-day PHE volume and clinical outcome. However, this association might be driven by the hematoma volume, and it remains unclear whether PHE independently influences clinical outcome.
Previous studies showed increased MMP-9 levels in patients who developed hematoma expansion and neurological deterioration after ICH.26,27 Increased MMP-3 levels on admission were reported to be associated with mortality in ICH patients, both MMP-3 and MMP-9, with the residual scar volume at 3 months.27 Our study confirmed a significant role of the MMPs and VEGF, vascular endothelial growth factor.29,30 CE is defined as a premorbid cellular process, which can either be reversed or develop into necrotic cell death.29 However, the existence of CE in ICH is controversially discussed.5,7,30–33 Some previous DWI studies showed that ADC values increased globally in the perihematomal region.33 This is in line with our data. We detected CE in a similar proportion of patients (61%) on day 3. Instead of an association of CE with the MMP and GF levels, we found that CE was highly correlated with the hematoma volume. We therefore hypothesize that this secondary brain cell injury is likely a consequence of the primary mass effect, or caused by cytotoxic molecules, which are released from the clot or the destroyed brain parenchyma.

VEGF and Ang-1 levels were found to be related to BBB disruption and brain edema in animal models of ICH and brain injury.18–20 In a clinical ICH study, high levels of VEGF and Ang-1 at 72 hours after symptom onset were associated with good functional outcome and reduced lesion volume.21 In our study, neither VEGF nor Ang-1 were associated with PHE or clinical outcome. This could be a result of the different time points of GFs assessment.

Our study has some limitations. Overall, the sample size is small. However, considering the fact that MRI was used, and thus the best available method to quantify PHE,26,34 this study includes a relatively high number of cases. Second, our findings cannot be generalized to patients with coma on admission and to those noneligible for MRI. Particularly in patients with large hematomas who were not eligible for this study according to the exclusion criteria, pronounced CE and larger PHE volume can be expected. Third, we only investigated molecular marker levels on baseline, although after ICH significant changes over time were reported.27

In conclusion, our data show that MMP-3 levels are associated with perihematomal edema in acute spontaneous ICH patients. Increased levels of MMP-3 and MMP-9 are independently associated with poor clinical outcome. Further investigations are needed to explain the mechanisms behind them, which might lead to treatment options for the prevention of secondary brain damage and unfavorable outcome.

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Disclosures
None.

References


Association of Molecular Markers With Perihematomal Edema and Clinical Outcome in Intracerebral Hemorrhage
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