Early Prediction of Gross Hemorrhagic Transformation by Noncontrast Agent MRI Cluster Analysis After Embolic Stroke in Rat

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Background and Purpose—Our goal was to develop magnetic resonance indices, without image contrast agent enhancement, that predict hemorrhagic transformation (HT) in a rat model of embolic stroke.

Methods—Male Wistar rats subjected to embolic stroke with (n=12) or without (n=10) the combination treatment with recombinant tissue plasminogen activator and an anti–platelet glycoprotein IIb/IIIa antibody 7E3 F(ab')2 initiated at 4 hours after onset of stroke were investigated using a 7-T MRI system. Radiofrequency saturation T1 (T1sat) maps with magnetization transfer, apparent diffusion coefficient of water (ADCw) maps in 3 directions, and T2 maps were measured at 2, 24, and 48 hours after embolization. MRI data were analyzed individually and using 2D cluster plots. Histological measurements were obtained at 48 hours.

Results—Gross hemorrhage was detected at 48 hours in 7 (4 control, 3 treated) of 22 animals. The 2D cluster plot using MRI T1sat and ADCw maps obtained at 2 hours after stroke predicted all gross HT. The location of gross hemorrhage predicted by the 2D cluster plot was within 0.75 mm of the identifying MRI cluster.

Conclusions—The 2D MRI cluster plot analysis using T1sat and ADCw maps acquired at 2 hours after the onset of embolic stroke predicts gross HT. (Stroke. 2005;36:1247-1252.)

Key Words: blood–brain barrier ■ magnetic resonance imaging ■ stroke

The incidence of symptomatic hemorrhagic transformation (HT) during the first 36 hours after the onset of stroke is significantly higher in patients receiving tissue plasminogen activator (tPA) than in placebo-treated patients (0.6% versus 6.4%), and 61% of the patients with symptomatic HT died within 3 months.1 A method to assess the risk of HT in ischemic cerebral tissue after stroke would improve the safety of thrombolytic therapy and increase its utilization.

No precise predictors of HT have been identified. Computed tomography (CT) can diagnose hemorrhage once it has occurred;2 however, it cannot predict HT unless high-dose contrast-enhanced CT is used.3–4 MRI with contrast obtained at 2 to 3 hours after middle cerebral artery occlusion (MCAo) in rat has been correlated with HT.5–7 In the present study, a 2D cluster plot method that uses maps of the trace of the apparent diffusion coefficient of water (ADCw) in brain tissue and saturation T1 (T1sat) of cerebral tissue, without the use of contrast, is shown to localize and to predict HT regions in a rat model of embolic stroke with a high degree of specificity.

Materials and Methods

All studies were performed in accordance with institutional guidelines for animal research under a protocol approved by the institutional animal care and use committee of Henry Ford Hospital.

Animal Model and Experimental Groups

Male Wistar rats (weighing 300 to 350 g) subjected to embolic stroke8 were divided randomly into 2 groups: with (n=12) or without (n=10) the combination treatment with tPA and platelet glycoprotein IIb/IIIa (GPIIb/IIIa) inhibitor at 4 hours after stroke. The F(ab')2 fragment of the murine monoclonal antibody 7E3, which reacts with GPIIb/IIIa, was given intravenously at a bolus dose of 6.0 mg/kg 4 hours after embolic MCAo and was followed by a second intraperitoneal dose (6.0 mg/kg) at 12 hours after the first dose. Recombinant tPA (rtPA) was infused intravenously at a total dose of 10 mg/kg (10% bolus 4 hours after ischemia and the remainder at a continuous infusion over a 30-minute interval). All animals were euthanized at 48 hours after MCAo.

MRI on 7T System

MRI measurements were performed using a 7-T, 20-cm bore magnet interfaced to a Bruker console, with a 12-cm bore actively shielded gradient coil set capable of producing a magnetic field gradient up to 200 mT/m. A birdcage radiofrequency (RF) coil was used as the...
transmitter and a surface coil as the receiver. During MRI measurements, stereotaxic ear bars were used to minimize movement and the anesthesia was maintained using a gas mixture (69% N2O, 30% O2, and 1% halothane). Rectal temperature was kept at 37±1.0°C. The right femoral artery and vein were cannulated for monitoring blood pressure, blood gas, and drug administration, respectively.

A tripilot imaging sequence was used for reproducible positioning of the animal in the magnet at each MRI session. A set of magnetic resonance (MR) images, including diffusion-weighted imaging (DWI), multiecho T2 measurement, and T1sat with magnetization transfer measurements, was performed before ischemia, repeatedly from 1 hour to 3 hours after the onset of embolization, and at 24 hours, as well as 48 hours after embolization for all animals. Quantitative estimates of tissue T1sat values were generated using an imaging variant of the Look-Locker technique. Two continuous wave saturation RF pulses are inserted into the Look-Locker sequence, with the first inserted immediately before the inversion pulse and lasting 4.5 s, and the second 40-ms long, inserted after the signal acquisition. The offset frequency of saturation pulses is 8 kHz, and the rotational frequency of B1 field is 0.5 kHz. Inversion of the longitudinal magnetization was accomplished using a nonelective hyperbolic secant adiabatic pulse of 8-ms duration. One phase-encode line of 32 small-tip angle-gradient echo images (echo time [TE] of 2.2 ms) was acquired at 80-ms intervals after each inversion. The total recovery time (TR) was 11 s. With this sequence, a slice of T1sat map was obtained within 12 minutes (32-mm field of view [FOV]; 128×64 matrix; 2-mm slice thickness).

The trace ADCw was measured in each x, y, and z direction. A 2D Fourier transform (2DFT) multislice spin-echo sequence (13 slices with 1-mm thickness; 32-mm FOV; 128×64 matrix; 1.5 s; TE 40 ms) was modified to include 2 10-ms diffusion-weighted gradient pulses, 1 on either side of the refocusing 180° RF pulse. A series of 3 images with gradient b-values of 0, 600, and 1200 s/mm2 were obtained. Total time for the entire 3-direction sequence was ~15 minutes.

T1sat was measured using a standard 2DFT multislice (13 slices with 1-mm thickness) multiecho (6 echoes) MRI. TEs were 20, 40, 60, 80, and 120 ms, and the TR was 0.7 s. Images were produced using a 32-mm FOV and 128×64 matrix. The total time for the entire sequence was ~9 minutes.

### Histological Measurement of Hemorrhage

After the final MRI measurement, animals were anesthetized with ketamine (44 mg/kg IP) and xylazine (13 mg/kg IP) and were transcardially perfused with heparinized saline followed by 10% neutral buffered formalin. The head was immerged in formalin solution for 1 hour, after which a total of 7 2-mm-thick blocks of brain tissue were cut, processed, and embedded in paraffin. Coronal sections (6-μm thick) were cut from each block and stained with hematoxylin and eosin (H&E) for evaluation of ischemic cell damage and hemorrhage.

Gross hemorrhage was defined as blood evident to the unaided eye on the H&E-stained sections and confirmed by microscopy. Microscopic hemorrhage was defined as blood evident only by microscopy (>20).

Each H&E-stained section was evaluated at ×10 magnification. The area of hemorrhage was measured by tracing the areas for each section on the computer monitor screen.

### 2D Cluster Plot Analysis

All MR images were reconstructed using a 128×128 matrix. The different MRI maps and histological section images were coregistered and analyzed using “Eigentool” software. The ADCw and T1 maps were produced on a pixel-by-pixel basis using a linear least-square fitting. A modified nonlinear optimization procedure was used to estimate the T1sat parameter.

MRI maps of cerebral tissue T1sat and ADCw, as well as T2, were analyzed as a 2D cluster plot to segment the cerebral tissue into normal area and ischemic area with and without brain–blood barrier (BBB) disruption. The individual tissue signatures identified from the various clusters were then transposed back onto the original images (eg, T1sat map) for anatomical reference and measurements of abnormal regions. At 2 hours after the MCAo, the area for which ADCw value is below the ADCw threshold of mean−2SD of the contralateral cerebral tissue was identified as the ischemic area. Within the ischemic area, the ADCw value of the mean−SD of the ischemic cerebral tissue was designated as the ADCw threshold value for discriminating hemorrhage. All T1sat maps exhibited a set of values that naturally separated into different groups. The hemorrhagic threshold for T1sat was selected among the distinct sets of values so that the T1sat values of normal white and gray matter were below the threshold. The area for which T2 value is above the T2 threshold of mean+2SD of the contralateral cerebral tissue was identified as abnormal tissue. These thresholds were used alone in ADCw, T1sat and T2 maps, respectively, as well as combined in 2D cluster plots (T1sat−ADCw or T2−ADCw). Measurements of MRI parameters were classified as ischemic values when measured within the ischemic region but outside of the hemorrhagic region. Measurements with the regions of gross HT identified by the 2D cluster plot (T1sat−ADCw) were classified as hemorrhagic values.

MRI cluster–defined HT areas were compared with the HT areas from histology. Observations are summarized as mean±SD.

### Results

Figure 1A presents a 2D cluster plot of T1sat (Figure 1B) versus ADCw (Figure 1C) maps, acquired at 2 hours after onset of ischemia, from a rat treated with rtPA and 7E3 F(ab′)2, administered at 4 hours after onset of embolic stroke. The structure of the 2D cluster plot along the T1sat axis naturally separated the clusters into several parts, shown in Figure 1D. These various clusters, according to their T1sat values, were transposed back onto the original image as a theme image, as shown in Figure 1E. Clusters with T1sat <391 ms were scattered like noise and were ignored. T1sat >807 ms were identified as cerebrospinal fluid and pixels within ventricles. Clusters with 391 ms < T1sat < 524 ms and 524 ms < T1sat < 658 ms encompassed the normal white matter and gray matter, respectively. From the theme image, we assume that the clusters with 658 ms < T1sat < 807 ms contained abnormal cerebral tissue, as well as partial volume of ventricle pixels and noise adjacent to the ventricles or along the brain boundary (Figure 1E, red).

A sharp decrease was present in the acute ADCw map acquired at 2 hours after embolic MCAo (Figure 1C). Figure 1F presents the back-transposed image by cluster with the threshold of ADCw <6.0×10−4 s/mm² only.

Using the combined conditions, 658ms < T1sat < 807 ms and ADCw <6.0×10−4 s/mm² (Figure 1G, red box), the cluster clearly identifies 3 regions (excluding the 3 single pixels) noted in red within the coronal section of cerebral tissue, as shown in Figure 1H. Comparing this MRI map (Figure 1H) with the histological section of rat brain at 48 hours after onset of embolic MCAo, shown in Figure 1I, the 3 regions identified by the 2D cluster plot using acute (2-hour) T1sat and ADCw maps accurately predicted the gross HT areas in size and location inside of the rat brain at 48 hours after stroke without any imaging contrast agent intervention. Similar and complementary data are shown for 2 additional representative rats in Figure 2.

This result is also apparent in control rats subjected to embolic stroke without treatment. Using H&E staining, we found that 4 control rats and 3 treated rats showed gross hemorrhage. All animals exhibit the similar natural separation...
along $T_{1sat}$ in the 2D cluster plots but at different values. For all rats with gross hemorrhage, the acute $T_{1sat}$-ADC$_w$ 2D cluster plot using individual threshold values predicts regions of gross hemorrhage histologically measured at 48 hours. The Table lists the MRI measurements at 2 hours after stroke for hemorrhagic size and relative errors compared with the histological measurement of hemorrhage at 48 hours after stroke. The histologically measured areas of hemorrhage for all rats are 0.30±0.27 mm$^2$, whereas the corresponding $T_{1sat}$-ADC$_w$ 2D cluster plot areas are 0.80±0.55 mm$^2$. The $T_{1sat}$-ADC$_w$ 2D cluster plot overestimated the area of gross hemorrhage in 6 of 7 rats with gross HT. $T_{1sat}$ map alone and ADC$_w$ map alone measured gross hemorrhage as 18.9±6.3 mm$^2$ and 11.6±9.5 mm$^2$, respectively. Thus, the errors of measurement of areas of gross hemorrhage with $T_{1sat}$ or ADC$_w$ maps, individually, far exceed those of the 2D

Figure 1. A 2D cluster plot (A) of $T_{1sat}$ map (B) vs ADC$_w$ map (C) from a rat treated with rtPA and 7E3 F(ab')$_2$ administered at 4 hours after onset of embolic stroke. The structure of the 2D-image plot along the $T_{1sat}$ axis naturally separated into clusters (D). These various clusters, according to their $T_{1sat}$ values, were transposed back onto the original image, as a theme image (E). The theme image (F) used a restricted cluster with ADC$_w$ < 6.0×10$^{-4}$ s/mm$^2$. Using the combined conditions, 658 ms < $T_{1sat}$ < 807 ms and ADC$_w$ < 6.0×10$^{-4}$ s/mm$^2$ (G), the cluster would identity 3 regions (H), reflecting gross HT measured in the histological section of rat brain at 48 hours after onset of embolic MCAo (I). The 2D cluster plot (J), using acute $T_2$ (K) and ADC$_w$ maps, also identified the gross HT areas in size and location inside the rat brain (L).
cluster plot. The positions of gross HT tissue between $T_{1\text{sat}}-\text{ADC}_w$ 2D cluster plot and histological measurements were within 3 pixels ($\approx 0.75 \text{ mm}$). We used the 2D cluster plot using $T_2$ and ADC$_w$ maps in the same animal shown in Figure 1A through 1I. Figure 1J shows the 2D cluster plot of the $T_2$ map (Figure 1K; acquired at 2 hours after MCAo) versus ADC$_w$ map of the central slice. With the thresholds of $T_2 > 72 \text{ ms}$ and ADC$_w < 6.0 \times 10^{-4} \text{ s/mm}^2$, a similar result to $T_{1\text{sat}}-\text{ADC}_w$ 2D cluster plot was obtained by $T_2-\text{ADC}_w$ 2D cluster plot, as shown in Figure 1L. The areas of gross HT predicted by $T_2-\text{ADC}_w$ 2D cluster plot are $1.04 \pm 1.03 \text{ mm}^2$. These data show that $T_{1\text{sat}}-\text{ADC}_w$ cluster analysis more accurately identifies tissue destined for HT than the $T_2-\text{ADC}_w$ cluster analysis. For the $T_{1\text{sat}}-\text{ADC}_w$ 2D cluster plot, there were no false positives (ie, signal and no gross hemorrhage), as well as no false negatives (ie, gross hemorrhage and no 2D cluster signal). The $T_2-\text{ADC}_w$ 2D cluster plot were noisier than the $T_{1\text{sat}}-\text{ADC}_w$ plot and exhibited scattered false-positive signals.

The 2D cluster plot approach failed to detect regions (scattered noisy pixels) confirmed histologically as microscopic hemorrhage at 48 hours in by H&E-stained coronal sections. Furthermore, the 2D cluster plot using 24- and 48-hour MRI maps failed to identify the gross hemorrhagic region histologically measured at 48 hours after stroke.

The quantitative analyses of MRI parameters are shown in Figure 3. The relative $T_{1\text{sat}}$ values of hemorrhagic and ischemic regions to normal regions of the contralateral tissue (Figure 3A) indicated that the hemorrhagic $T_{1\text{sat}}$ was significantly higher than the ischemic $T_{1\text{sat}}$ at 2 hours after the onset of embolic stroke. But this significant difference disappeared at 24 hours after stroke onset. The evolution of ADC$_w$ for ischemic and hemorrhagic tissue is shown as Figure 3B. A significant difference in relative ADC$_w$ was detected at 2 hours after stroke between hemorrhagic and ischemic tissue. The differences of relative ADC$_w$ values between the hemorrhagic and ischemic regions to normal tissue lost its significance at 24 hours.

The $T_2$ map behaves similarly to the $T_{1\text{sat}}$ map (Figure 3C), although the relative $T_2$ values of hemorrhagic and ischemic regions to the normal region of the contralateral side only had marginally significant differences at 2 hours after the onset of embolic stroke.

### Discussion

In the present study, we show that MRI without contrast agent, as early as 2 hours after the onset of embolic stroke, an objective 2D cluster plot analysis using $T_{1\text{sat}}$ and ADC$_w$ maps, predicts gross HT present 48 hours after stroke. This suggests that MRI can be used to predict gross HT early (2 hours after stroke onset) by its intrinsic contrast mechanisms.

The 2D cluster plot consists of 2 MRI parameters: $T_{1\text{sat}}$ and ADC$_w$ that respond early to stroke. The $T_{1\text{sat}}$ is related to the apparent forward transfer rate of magnetization between macromolecular protons and free water protons, which is sensitive to BBB disruption in the rat model of embolic stroke. Our data demonstrated that $T_{1\text{sat}}$ within the region destined for gross HT increased early (at 2 hours after the onset of embolic stroke). DWI has been used in the early diagnosis of stroke. Threshold values of ADC$_w$ obtained acutely after MCAo may identify and distinguish tissue destined to be reversibly or irreversibly damaged and may reflect BBB damage. Our data also show a sharp decrease of ADC$_w$ in the area destined for gross HT. The 48-hour histological HT regions colocalize to the area with ADC$_w$ values $< 6.0 \times 10^{-4} \text{ mm}^2/\text{s}$ at 2 hours after MCAo.

Figure 1E and 1F and the Table show that a single parameter of $T_{1\text{sat}}$ or ADC$_w$, respectively, identifies larger and more diffuse areas than the HT measured at 48 hours (Figure 1I). These errors were much $> 349 \pm 430\%$ of the 2D cluster plot that only combined the $T_{1\text{sat}}$ and ADC$_w$ parameters.

Comparing the different temporal profiles of ischemic and gross HT regions with $T_{1\text{sat}}$ and ADC$_w$ maps, it appears that the gross HT region is a specific area within the ischemic tissue where the ischemic lesion developed rapidly. The quantitative results of $T_2$ map show areas with increased $T_2$ value at 2 hours after embolic MCAo, although we expect that $T_2$ will generally not be altered within 2 hours after stroke onset. These data indicate that the $T_2$ change was marginally

### Table 1

<table>
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<th>Parameter</th>
<th>Histology</th>
<th>$T_{1\text{sat}}$-ADC$_w$</th>
<th>$T_2$-ADC$_w$</th>
<th>$T_{1\text{sat}}$</th>
<th>ADC$_w$</th>
<th>$T_2$</th>
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<td>Size, mm$^2$</td>
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<td>0.80±0.55</td>
<td>1.04±1.03</td>
<td>18.9±6.3</td>
<td>11.6±9.5</td>
<td>13.3±3.5</td>
</tr>
<tr>
<td>Error, %</td>
<td>...</td>
<td>349±430</td>
<td>573±616</td>
<td>9656±9739</td>
<td>4069±3933</td>
<td>857±6964</td>
</tr>
</tbody>
</table>

**Figure 2.** The 48-hour histological section revealed a large hemorrhage (B; arrow). This HT was predicted by the 2D plot of $T_{1\text{sat}}$-ADC$_w$ at 2 hours after onset of ischemia (A). For another animal with a small HT identified histologically at 48 hours (D; arrow), the HT was predicted by the 2-hour $T_{1\text{sat}}$-ADC$_w$ 2D plot analysis (C).
significant ($P<0.07$), unlike acute $T_{1\text{sat}}$ ($P<0.001$) or $ADC_w$ ($P<0.05$). As Figure 3D shows, the $T_1$ value did not exhibit a significant difference between the ischemic and hemorrhagic regions at 2 hours.

It is important to note that the early prediction of HT by 2D cluster plot using $T_{1\text{sat}}$ and $ADC_w$ maps acquired at 2 hours after the onset of stroke preceded any treatment. Thus, the combination treatment with rtPA and 7E3 F(ab’)$_2$ did not alter prediction of gross HT. These data suggest that treatment at 4 hours after stroke onset with rtPA and 7E3 F(ab’)$_2$ to the embolic rats does not increase the risk of gross HT at 48 hours after stroke. These data are consistent with our previous findings. Because all gross hemorrhage were predicted by cluster analysis at 2 hours, this implies that changes in tissue that lead to HT arise before 2 hours after the onset of stroke.

The 2-hour $T_{1\text{sat}}$–$ADC_w$ 2D cluster plot analysis fails to predict microscopic HT measured at 48 hours after stroke. Quantitative $T_{1\text{sat}}$ cluster analysis was first proposed by Nagesh et al to identify and discriminate different pathologies in a model of transient focal cerebral ischemia in the rat. The tissue changes associated with microscopic hemorrhagic may be below the resolution for MRI. As a result, the MRI parameters failed to discriminate microscopic hemorrhagic within the ischemic lesion area at 2 hours after the onset of stroke. The inability to predict microscopic HT may also be related to the time of onset of HT. Thus, tissue changes leading to microscopic HT may occur after 2 hours, which results in image enhancement with contrast agent at 24 hours after stroke onset.

MRI parameters including $T_{1\text{sat}}$ and $ADC_w$ were unable to significantly discriminate differences between gross HT and ischemic regions at 24 hours after onset of ischemia. The $T_{1\text{sat}}$ of ischemic tissue increased, approaching the $T_{1\text{sat}}$ of gross HT tissue, whereas the $ADC_w$ value of ischemic tissue surpassed that of gross HT tissue. Thus, the 2D cluster plot using $T_{1\text{sat}}$ and $ADC_w$ maps acquired at 24 hours failed to identify any gross HT region, as well as microscopic HT region. The exact time point at which the $T_{1\text{sat}}$ and $ADC_w$ cluster analysis fails to predict gross HT remains to be identified.

In summary, our data demonstrated that an objective 2D MRI cluster plot analysis using $T_{1\text{sat}}$ and $ADC_w$ maps acquired at 2 hours after the onset of embolic stroke can precisely predict, with no false-negative or false-positive data, gross HT for the control group and the group treated with the combination of rtPA and 7E3 F(ab’)$_2$. This method does not require contrast agent. Our data also confirm that the intervention of combination treatment at 4 hours after the onset of embolic stroke with rtPA and 7E3 F(ab’)$_2$ does not increase the HT risk by 48 hours compared with the treatment with saline.

**Acknowledgments**

This work was supported by National Institute of Neurological Disorders and Stroke grants PO1 NS23393, RO1 NS38292, RO1 NS43324, and HL64766.

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Stroke. 2005;36:1247-1252; originally published online May 5, 2005;
doi: 10.1161/01.STR.0000166199.10017.c5

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