When we divided the patients into two groups whose volume reduction at PCC was less than ($n = 9$) or greater than ($n = 11$) the average (difference in fractional volume change = −0.05), we found a significant difference in the fractional change of scored apathy between the groups (less-than-average group, change = −0.17; greater-than-average group, change = 0.43; $t = 2.41$; $p = 0.03$). When we considered the confounding effects of age, sex, laterality of the infarction, and acute stroke size as covariates in a one-way analysis of covariance the change in the apathy scores between groups showed a trend that did not reach significance ($F_{1, 14} = 3.2$, $p < 0.1$).

## Discussion

In the present study, we found a significant volume reduction in the anterior part of the PCC over the 6 months following a stroke, by exploratory VBM analysis. Furthermore, the fractional volume change was observed to be negatively correlated with the apathy scale scores during the 6 months after the stroke. The reduced volume of PCC due to the 6-month interval was associated with increased apathy scores. Our findings indicate that the neuronal changes in PCC after stroke are one of the factors that affect the degree of poststroke apathy.

In the patients, delayed atrophy was observed in a part of the PCC anatomically remote from the respective subcortical infarct site. This finding may reflect degeneration secondary to neuronal loss, possibly due to Wallerian degeneration, which is a degeneration of distal parts of nerve axons after injury of the proximal axon or cell body (Thomalla *et al.*, 2004). Axonal degeneration, in turn, leads to the death of postsynaptic cell bodies (Raff *et al.*, 2002) and should result in a secondary volume reduction in the part of the brain constituting the projection target of the lost axons. From an anatomical perspective, the PCC has dense structural connections to many other brain regions, suggesting a role as a structural hub (Hagmann *et al.*, 2008). Its volume reduction seen here may reflect the simultaneous loss of multiple afferent projections due to stroke in spatially different sites.

We found more apathetic change in the group of stroke patients with larger volume reductions in PCC. A recent meta-analysis showed that apathy occurred in almost every third patient after a stroke (van Dalen *et al.*, 2013). Because poststroke apathy can have a negative effect on the rehabilitation of activities of daily living or quality of life (Samus *et al.*, 2005; Hama *et al.*, 2007), poststroke apathy has attracted considerable attention. Marin has described apathy as a neuropsychiatric syndrome characterized by diminished goal-directed overt behavior, diminished goal-directed cognition, and diminished emotional concomitants of goal-directed behavior (Marin, 1991).

How is the volume reduction of PCC related to the increase in apathy of stroke patients? The PCC is a hub within the brain, connecting networks that function together to support complex behavior (Hagmann *et al.*, 2008). Leech *et al.* (2011) reported that the PCC is subdivided into dorsal and ventral parts differing in the regions to which they functionally connect. The dorsal part of the PCC is consistent with the region showing the volume reduction in stroke patients in the present study. Anatomically, the dorsal part of the PCC has connectivity with the ventral medial prefrontal cortex, part of the default mode network (DMN), and frontal and parietal regions involved in the cognitive control network (CCN). Abnormalities have been identified in CCN and DMN during episodes of late-life depression that have often been characterized as apathy (Alexopoulos *et al.*, 2012), and the PCC was suggested to have an important role in the regulation of these two networks necessary for controlling efficient goal-directed behavior (Leech *et al.*, 2011). We speculate that the degeneration of the PCC following a stroke might impair the function of control of goal-directed behavior, leading to increased apathy scores.

Our study has some limitations. First, we could not find volume reductions in any other regions of the brain than the PCC. However, the sample size in our study was not large enough to reveal moderate-sized differences between groups. Further study with increased numbers of subjects will be necessary for drawing any definitive conclusions. Second, we could not find associations between cognitive scales and the volume reduction at PCC. We assessed cognitive deficits using MMSE scores, but this test is not highly sensitive to differences between persons with normal and higher performances; thus, the possible presence of a ceiling effect must be considered. Extensive neuropsychological testing is needed for the assessment of cognitive dysfunction. Finally, we were not able to control precisely for important variables, such as social support, premorbid personality, and prior medication histories. Further analysis in light of these points is needed to confirm our present findings.

## Conclusion

The present study suggests that delayed atrophic change in the PCC is evident 6 months after a stroke. We also found that the fractional volume change during the 6 months following a stroke was negatively correlated with apathy scale scores. The larger the volume reduction in PCC, the

greater was the increase in apathy-scale scores. The delayed atrophy of part of the PCC seen here may reflect degeneration secondary to neuronal loss due to stroke. Damage in this area may damage control of goal-directed behavior, and it is plausible that such a defect would appear clinically as greater apathy. Knowledge of secondary brain degeneration in stroke patients and its impact on the adverse outcome of development of apathy could provide clues for recognizing new therapeutic targets.

## Conflict of interest

None declared.

Key points

- We found significant volume reduction in the anterior part of the PCC over 6 months after the incidence of stroke in patients by exploratory VBM analysis.
- We also found that the rate of volume change was significantly correlated with the apathy scale scores during 6 months post-stroke.
- The delayed atrophy of the PCC may reflect degeneration secondary to neuronal loss due to stroke, and it might deteriorate the function of controlling goal-directed behavior related to apathetic change.

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# Microstructural abnormalities in white matter and their effect on depressive symptoms after stroke

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ABSTRACT

The aim of the study was to investigate the existence of microstructural abnormalities in the white matter of the brain in stroke patients, as well as the relationship between these microstructural abnormalities and changes in depressive symptoms over 6 months. Participants were 29 acute ischemic stroke patients and 37 healthy control subjects. Depressive symptoms were assessed in all subjects using the Hamilton Rating Scale for Depression and the Zung Self-rating Depression Scale. Whole brain voxel-based analysis was used to compare diffusion tensor imaging measures of Fractional Anisotropy (FA) between the groups. Six-month follow-up examinations were conducted. Patients showed significantly lower white matter FA values in the left and right anterior limbs of the internal capsule, and 6 months after the stroke they showed significantly increased FA values in these regions. We found a significant negative correlation between the increased ratio of the FA values and the change in depression scale scores at 6-month follow-up. Regional white matter damage may reflect abnormalities in neuroanatomical pathways related to the pathophysiology of depression.



## 1. Introduction

Depression is the most common and serious emotional symptom following a stroke and is associated with excess disability, cognitive impairment and mortality (Whyte and Mulsant, 2002). Although there is no consensus about the relationship between lesion location and post-stroke depressive symptoms, Magnetic Resonance Imaging (MRI) studies have found a high prevalence of depressive symptoms in patients with lesions that affect structures of the prefronto-subcortical circuit (Vataja et al., 2001, 2004). Recent studies have highlighted the specific relevance of the Limbic-Cortical-Striatal-Pallidal-Thalamic (LCSPT) circuit in the pathophysiology of major depressive disorder (Drevets et al., 2008; Hasler et al., 2008) and of depression due to stroke (Terroni et al., 2011; Paradiso et al., 2013).

Diffusion Tensor Imaging (DTI) combines a conventional MRI sequence with additional magnetic field gradients to quantify water diffusion, namely, Fractional Anisotropy (FA), the degree to which diffusion is directionally hindered, which reflects the microstructural integrity of the white matter tracts. Microstructural damage to white matter tracts may confer a biological vulnerability to the onset of depressive symptoms in stroke patients. To our knowledge, however, no studies have investigated the existence of microstructural abnormalities of white matter in stroke patients and examined whether the diminution of microstructural abnormalities decreased the vulnerability to post-stroke depression, as measured by increases in depression scale scores in stroke patients that are noted before the onset of severe depression.

Thus, the primary aim of the present study was to investigate the existence of microstructural abnormalities in white matter tracts in stroke patients, as well as the relationship between the recovery from these microstructure abnormalities and the change in depression scale scores 6 months after a stroke. DTI was performed and whole brain voxel-based analysis was used to compare FA between groups of acute ischemic stroke patients

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and healthy control subjects. Six-month follow-up examinations were conducted. On the day of the MRI scan, depressive symptoms were evaluated with the Hamilton Rating Scale for Depression and the Zung Self-rating Depression Scale.

## 2. Methods

### 2.1. Participants

After complete description of the study to the subjects, written informed consent was obtained. The study was approved by the medical ethics committee of the National Cerebral and Cardiovascular Center in Japan. The patients were of Japanese ethnicity and were recruited from the neurology unit of the National Cerebral and Cardiovascular Center hospital. These patients had initially been hospitalized for treatment of acute ischemic stroke.

Stroke was diagnosed by neurologists according to World Health Organization (WHO) criteria. After the assessment, a group of psychiatrists and neurologists reviewed the data and reached a consensus regarding the presence or absence of psychiatric disease, including dementia, according to DSM-IV criteria. Patients were included if they met the following criteria: (1) a focal lesion of either the right or left hemisphere on MRI; (2) absence of other neurological, neurotoxic, or metabolic conditions; (3) modest ischemic insult (modified Rankin scale $\leq 4$) with absence of a significant verbal comprehension deficit; and (4) occurrence of stroke 10–28 days before the examinations. Exclusion criteria were as follows: (1) transient ischemic attack, cerebral hemorrhage, subdural hematoma or subarachnoid hemorrhage; (2) history of a Central Nervous System (CNS) disease such as tumor, trauma, hydrocephalus, and Parkinson's disease; and (3) pre-stroke history of depression. Thirty-eight patients who volunteered to participate in the study were screened for eligibility. We excluded 5 subjects who did not meet the study criteria. In addition, four patients had not completed the MRI scan due to fatigue. A final group of 29 patients met the criteria and participated in this study.

Thirty seven healthy volunteers were recruited from the local area by poster advertisement. Exclusion criteria for the volunteers were a history or present diagnosis of any DSM-IV axis I or any neurological illness. Major characteristics of this cohort are summarized in Table 1. To reliably elucidate differences in white matter integrity between groups, the target total sample size was set at above 52, which was expected to yield power $\geq 0.8$, based on $\alpha \leq 0.05$ and assuming a large effect size ($f=0.4$) with the analysis of covariance (ANCOVA) used in this study (Cohen, 1977), and the sample size of this study met the power requirement.

All patients and volunteers were assessed with a series of standardized, quantitative measurements of depressive symptoms [Hamilton Rating Scale for Depression (HAM-D) (Hamilton, 1960), Zung Self-rating Depression Scale (SDS) (Zung, 1965)] and cognitive function [Mini-Mental State examination (MMSE) (Folstein et al., 1975)] on the day of the MRI scan. A neurological examination [modified Rankin scale: mRS (Brott et al., 1989)] was also carried out in the patients. MRIs were conducted for all of the subjects.

Six-month follow-up MRI examinations were also conducted for 18 of 29 patients and 19 of 37 healthy subjects. The other patients and controls were lost to follow-up because we were unable to contact them at 6 months after the first study or they declined to further participate in this study due to health problems, business, feeling of rejection, and so on. On the day of the follow-up MRI scan, the participants underwent the same battery of depressive, cognitive function and (for the patients) neurological measurements that had been performed at the time of the initial MRI. There were no changes in medication between baseline and follow-up. No patients and healthy subjects were diagnosed as meeting DSM-IV criteria for major depression on the day of the initial MRI. Two patients were diagnosed as meeting criteria for major depression for the first time on the day of the follow-up MRI, and they were prescribed medication after the examinations. No patients were on antidepressant treatment during the examinations.

### 2.2. MRI acquisition

All MRI examinations were performed using a 3-T whole-body scanner (Signa Excite HD V12M4; GE Healthcare, Milwaukee, WI, USA) with an eight-channel phased-array brain coil. DT images were acquired with a locally modified single-shot Echo-Planar Imaging (EPI) sequence by using parallel acquisition at a reduction (ASSET) factor of 2, in the axial plane. Imaging parameters were as follows: repetition time (TR)=17 s; echo time (TE)=72 ms; $b$=0, 1000 s/mm$^2$; acquisition matrix, 128 × 128; field of view (FOV), 256 mm; section thickness, 2.0 mm; no intersection gap; 74 sections. The reconstruction matrix was the same as the acquisition matrix, and 2 mm × 2 mm × 2 mm isotropic voxel data were obtained. Motion Probing Gradient (MPG) was applied in 55 directions, the number of images was 4144, and the acquisition time was 15 min, 52 s.

To reduce blurring and signal loss arising from field inhomogeneity, an automated high-order shimming method based on spiral acquisitions (Kim et al., 2002) was used before acquiring DTI scans. To correct for motion and distortion from eddy current and B0 inhomogeneity, FMRIB software (FMRIB Center, Department of Clinical Neurology, University of Oxford, Oxford, England; http://www.fmrib.ox.ac.uk/fsl/) was used. B0 field mapping data were also acquired with the echo time shift (of 2.237 ms) method based on two gradient echo sequences.

High-resolution three-dimensional T1-weighted images were acquired using a spoiled gradient-recalled sequence (TR=12.8 ms, TE=2.6 ms, flip angle=8°, FOV, 256 mm; 188 sections in the sagittal plane; acquisition matrix, 256 × 256; acquired resolution, 1 × 1 × 1 mm). T2-weighted images were obtained using a fast-spin echo (TR=4800 ms; TE=101 ms; echo train length (ETL)=8; FOV=256 mm; 74 slices in the transverse plane; acquisition matrix, 160 × 160, acquired resolution, 1 × 1 × 2 mm).

**Table 1**
Demographic characteristics of patients and healthy control subjects.

| Characteristic | Stroke patients ($n$=29) | Healthy controls ($n$=37) | $t_{64}$ or $X^2$ | $P$ |
|---|---|---|---|---|
| Age (years) | 68.7 ± 8.2 | 67.5 ± 5.2 | $t$=0.77 | 0.45 |
| Female sex ($n$, %) | 6 (20.7) | 15 (40.5) | $\chi^2$=2.95 | 0.10 |
| MMSE score | 27.8 ± 3.0 | 29.2 ± 1.0 | $t$=2.45 | 0.02* |
| SDS score | 26.5 ± 5.6 | 24.1 ± 3.6 | $t$=2.03 | 0.05* |
| HAM-D score | 2.6 ± 2.5 | 1.1 ± 1.8 | $t$=2.64 | 0.01* |
| mRS score | 2.2 ± 0.8 | – | | |
| Number of acute infarcts | 1.2 ± 0.6 | – | | |
| Volume of acute infarcts (ml) | 2.0 ± 2.3 | – | | |
| Acute infarcts ($n$, %) in | | | | |
| Frontal cortex | 1 (3.4) | – | | |
| Occipital cortex | 1 (3.4) | – | | |
| Basal ganglia | 13 (44.8) | – | | |
| Thalamus | 4 (13.8) | – | | |
| Subcortical white matter infarcts in | | | | |
| Frontal lobe | 6 (20.7) | – | | |
| Parietal lobe | 1 (3.4) | – | | |
| Temporal lobe | 1 (3.4) | – | | |
| Occipital lobe | 1 (3.4) | – | | |
| Genu of internal capsule | 1 (3.4) | – | | |
| Total | 10 (34.5) | – | | |
| Laterality of acute hemisphere infarcts | | | | |
| Left hemisphere ($n$, %) | 17 (58.6) | – | | |

MMSE=Mini-Mental State Examination. SDS=Zung Self-Rating Depression Scale. HAM-D=Hamilton Rating Scale for Depression. DWMH= deep white matter hyperintensity. PVH=Periventricular hyperintensity. mRS=Modified Rankin Scale. Data are mean ± S.D. * $p < 0.05$.

### 2.3. Image processing

FMRIB software was used to generate FA maps and three eigenvalues ($\lambda 1$, $\lambda 2$, and $\lambda 3$) from each individual. First, brain tissue was extracted using the Brain Extraction Tool in FSL software. Brain maps for each of the 55 directions were eddy-corrected, subsequent to which FA values were calculated at each voxel using the FSL FMRIB Diffusion Toolbox.

Image preprocessing and statistical analysis were carried out using SPM8 software (Wellcome Department of Imaging Neuroscience, London, England). Each subject's echo planar image was spatially normalized to the Montreal Neurological Institute echo planar image template using parameters determined from the normalization of the image with a $b$ value of 0 s/mm$^2$ and the echo planar image template in SPM8. Images were resampled with a final voxel size of 2 × 2 × 2 mm$^3$. Normalized maps were spatially smoothed using an isotropic Gaussian filter (8-mm full-width at half-maximum).

### 2.4. Voxel-based analysis

Voxel-based analysis was performed using SPM8 software. FA maps were compared between patients and healthy subjects by ANCOVA with age and gender as covariates of no interest. We included age and gender as covariates because it has been reported that they affect the white matter integrity (Inano et al., 2011). Statistical inference was done with a voxel-level threshold of $p < 0.05$, after family-wise error correction for multiple comparisons, with a minimum cluster size of 50 voxels. The regional FA value was calculated by averaging the FA values for all voxels within the voxel of interest (VOI) corresponding to the cluster composed of

significant contiguous voxels in the above analysis. The same VOIs were applied to $\lambda_1-\lambda_3$ images, and $\lambda_1-\lambda_3$ values were extracted. Axial ($\lambda_1$) and radial diffusivity ($[\lambda_2+\lambda_3]/2$) were compared.

### 2.5. Statistical analysis

Group differences in demographic characteristics between patients and healthy controls were examined by unpaired $t$-test and Pearson $\chi^2$ test. To examine the group differences of FA values and axial/radial diffusivity in VOIs shown in the voxel-based analysis, we performed ANCOVA with age and gender as covariates. We included age and gender as covariates because they reportedly affect white matter integrity (Inano et al., 2011). Paired $t$-tests were performed to examine the changes in the mRS, MMSE, SDS, and HAM-D scores and the FA values of patients and controls during the 6-month period after their initial examinations. We computed Pearson's correlations to examine the relationship between FA values and depressive symptoms at the first assessment and at the 6-month follow-up assessment. Pearson's correlations were also used to examine the relationship between the change in depression scale scores and the ratio of the FA values (FA values at second vs. initial examination) in patients. To examine whether the ratio of the FA values was related to the change in depression scale scores (SDS and HAM-D scores at second minus initial examination), we performed a multiple regression analysis with the change in depression scale scores as the dependent variable and the ratio of the FA values as the independent variable, after adjustment for age and gender.

All statistical tests were 2-tailed and reported at $\alpha < 0.05$. Bonferroni correction was applied to avoid type I errors due to the multiplicity of statistical analyses. Statistical analysis of the data was performed using SPSS for Windows 19.0 (IBM Japan Inc., Tokyo, Japan).

## 3. Results

### 3.1. Demographic and clinical data

Table 1 summarizes the demographic and clinical characteristics of the participants. Patients differed significantly from healthy control subjects in MMSE, SDS and HAM-D scores. The MMSE score was lower, and SDS and HAM-D scores were higher, among the patients. Table 1 also shows the mRS score and the location and volume of the infarctions among the patients. The main locations of the infarctions were the basal ganglia (44.8%), the subcortical white matter in the frontal lobe (20.7%), and the thalamus (13.8%).

### 3.2. Between-group comparisons of FA values

In the voxel-based analysis of FA values, the patient and healthy control groups differed in white matter FA values in the left and right anterior limbs of the internal capsule [left anterior limb of internal capsule: $(x, y, z)=(-24, 16, 16)$, cluster voxel size=189, $T=6.41$; right anterior limb of internal capsule: $(x, y, z)=(16, 6, 10)$, cluster voxel size=756, $T=6.86$] (Fig. 1a). Table 2 shows the quantification of the differences in FA value and radial/axial diffusivity in these affected regions. These regions revealed decreased axial diffusivity but no change in radial diffusivity. When we added the MMSE, SDS, and HAM-D scores as covariates in the ANCOVA, the results did not change.

### 3.3. Change in FA values of patients over 6 months

There were no significant differences in demographic data between participants who were followed and those who were lost to follow-up, except the age of the healthy control groups (Table 3). Table 4 shows the changes in psychometric scores and FA values and axial/radial diffusivity over 6 months in the followed-up patients and controls. Healthy controls showed no significant change of FA values in the anterior limb of the internal capsule 6 months after the initial examination (Table 4). Patients showed significantly increased FA values in the anterior limb of the internal capsule 6 months after the infarction, although their FA

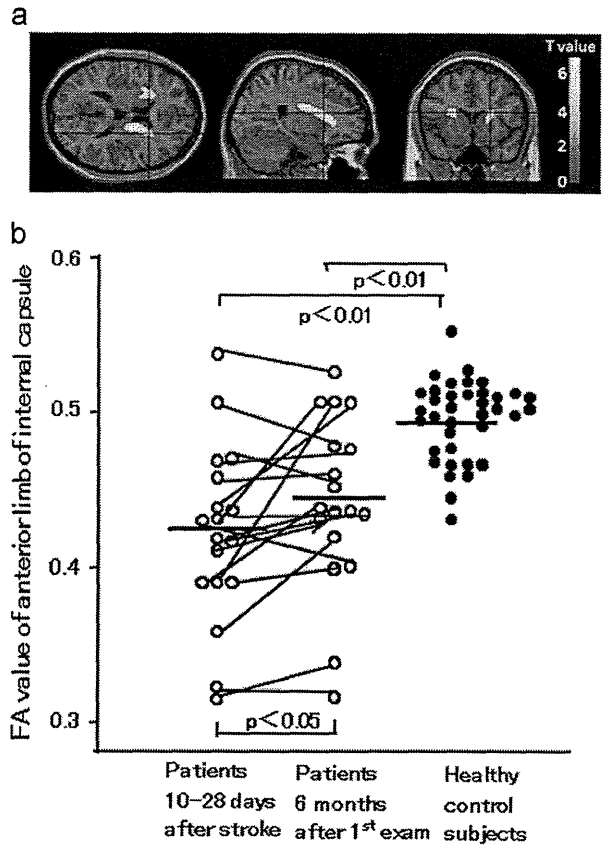


**Fig. 1.** White matter Fractional Anisotropy (FA) differences in voxel-based comparisons between stroke patients ($n=29$) and control subjects ($n=37$) (Fig. 1a), and scatter plots of FA values in the region of FA reduction among stroke patients ($n=18$) at 10–28 days after stroke/after 6-month follow-up and of control subjects ($n=37$) (Fig. 1b). (a) Images are presented in radiological orientation. Statistical parametric mapping projections were superimposed on a representative magnetic resonance image ($x=24, y=16, z=16$). Patients showed reduced FA in the right and left anterior limbs of the internal capsule. Statistical inferences were made with a voxel-level statistical threshold ($p < 0.05$) after family-wise error correction for multiple comparisons with a minimum cluster size of 50 voxels. (b) Significant increase in FA of the patients was observed at 6-month follow-up ($p < 0.05$), although the FA values of the patients were still lower than those of healthy subjects at both the initial and follow-up examinations ($p < 0.01$).

values were still lower relative to those of healthy control subjects at this time point (Table 4, Fig. 1b). There were no significant changes in MMSE, SDS and HAM-D scores 6 months after the initial examination in the groups of patients and healthy controls (Table 4).

There were no significant relationships between FA values and depressive symptoms at the first assessment and at the assessment performed 6 months later, but we found a significant negative correlation between the increased ratio of the FA values and the change in the depression scores on the SDS and HAM-D at 6-month follow-up ($r=-0.59$, $p=0.01$ for SDS; $r=-0.56$, $p=0.02$ for HAM-D) (Fig. 2). With Spearman's correlational analysis, the results were not changed ($r=-0.56$, $p=0.02$ for the SDS; $r=-0.73$, $p=0.001$ for the HAM-D). When we considered the influence of the volume of infarcts and lesion location [cortex ($n=2$), basal ganglia ($n=8$), thalamus ($n=1$), and subcortical white matter ($n=7$)] as a covariate in correlational analysis, the correlations were not changed ($r=-0.59$, $p=0.02$ for SDS; $r=-0.57$, $p=0.02$ for HAM-D). When we considered the influence of the degree of handicap by including the change in mRS scores as a covariate in the correlation analysis, which showed a significant decrease during follow-up (mRS score=$2.1\pm0.8$ at first examination, $1.6\pm0.6$ at 6-month follow-up, $t=4.12$, $p < 0.01$, paired

**Table 2**
Differences in values of FA and axial/radial diffusivity in VOIs between patients and healthy control subjects.

| FA and axial/radial diffusivity | Patients ($n=29$) | Healthy controls ($n=37$) | Analysis of covariance [a] | |
|---|---|---|---|---|
| | | | $F(1, 62)$ | $P$ |
| Left anterior limb of internal capsule | | | | |
| FA | $0.44 \pm 0.05$ | $0.49 \pm 0.05$ | 16.7 | $<0.001$** |
| Axial diffusivity ($\times 10^{-3}$) | $4.16 \pm 0.36$ | $4.37 \pm 0.35$ | 5.46 | 0.02 * |
| Radial diffusivity ($\times 10^{-3}$) | $3.94 \pm 0.33$ | $4.03 \pm 0.33$ | 1.38 | 0.25 |
| Right anterior limb of internal capsule | | | | |
| FA | $0.45 \pm 0.05$ | $0.51 \pm 0.05$ | 21.8 | $<0.001$** |
| Axial diffusivity ($\times 10^{-3}$) | $4.15 \pm 0.36$ | $4.36 \pm 0.36$ | 5.19 | 0.03 * |
| Radial diffusivity ($\times 10^{-3}$) | $3.91 \pm 0.33$ | $4.01 \pm 0.33$ | 1.38 | 0.24 |
| Average of right and left anterior limbs of internal capsule | | | | |
| FA | $0.44 \pm 0.05$ | $0.50 \pm 0.05$ | 20.8 | $<0.001$** |
| Axial diffusivity ($\times 10^{-3}$) | $4.15 \pm 0.36$ | $4.36 \pm 0.35$ | 5.35 | 0.02 * |
| Radial diffusivity ($\times 10^{-3}$) | $3.92 \pm 0.33$ | $4.02 \pm 0.33$ | 1.38 | 0.24 |

Data are mean ± S.D.

[a] Age and gender are entered as covariates.

* $p < 0.05$.

** $p < 0.01$.

**Table 3**
Demographic characteristics of patients and healthy control subjects who could be followed up and who were lost to follow-up.

| | Follow-up | Lost to follow-up | $t$ or $\chi^2$ | $P$ |
|---|---|---|---|---|
| *Patients* | $n=18$ | $n=11$ | | |
| Age (years) | $69.2 \pm 8.0$ | $67.6 \pm 9.0$ | 0.58 | 0.57 |
| Female sex ($n$, %) | 5 (26.3) | 1 (9.1) | 1.45 | 0.24 |
| mRS score | $2.1 \pm 0.8$ | $2.4 \pm 0.9$ | 1.00 | 0.33 |
| MMSE score | $27.6 \pm 3.5$ | $27.5 \pm 2.8$ | 0.47 | 0.64 |
| SDS score | $26.9 \pm 5.6$ | $27.1 \pm 7.3$ | 0.42 | 0.68 |
| HAM-D score | $3.1 \pm 2.7$ | $2.3 \pm 3.0$ | 0.46 | 0.65 |
| *Controls* | $n=19$ | $n=18$ | | |
| Age (years) | $69.1 \pm 8.0$ | $65.8 \pm 8.1$ | 5.22 | $<0.01$ |
| Female sex ($n$, %) | 10 (55.5) | 10 (55.5) | 0.03 | 0.86 |
| MMSE score | $29.2 \pm 1.2$ | $29.2 \pm 0.8$ | 0.10 | 0.92 |
| SDS score | $22.1 \pm 1.8$ | $26.2 \pm 1.8$ | 1.64 | 0.11 |
| HAM-D score | $0.4 \pm 0.6$ | $1.8 \pm 2.1$ | 1.61 | 0.12 |

$t$-test), the correlational results were unchanged ($r=-0.61$, $p=0.01$ for the SDS; $r=-0.58$, $p=0.02$ for the HAM-D).

With multiple regression analysis evaluating whether the increased ratio of FA values was related to the change in the SDS and HAM-D depression scores at 6-month follow-up, the ratio of FA values was negatively related to both the changes in the SDS scores (beta$=-0.44$, $p=0.03$) and the HAM-D scores (beta$=-0.46$, $p=0.04$).

## 4. Discussion

Our findings showed that stroke patients had lower FA in the bilateral anterior limbs of the internal capsule relative to healthy control subjects. Six months after onset, a significant increase in FA was noted, and it was associated with a reduction in depression scale scores. The association of the increase in FA and the reduction in depression scale scores remained significant when we considered the influence of the volume of infarcts and lesion location and the severity of neurological symptoms as a covariate in the partial correlation analysis.

The reduced FA level of patients was associated with decreased axial diffusivity. Axonal damage leads to a marked decrease in axial diffusivity, while demyelination leads to an increase in radial diffusivity (Song et al., 2005). Therefore, our finding was not a result of demyelination but rather of gross reduction in axonal number and/or size, possibly reflecting Wallerian degeneration secondary to neuronal loss due to stroke (Thomalla et al., 2004). From an anatomical perspective, the anterior limb of the internal capsule represents the intercept point in the course of the frontal-subcortical circuits (Axer and Keyserlingk, 2000), and it has extensive connectivity with the cortical and subcortical areas. Its reduced FA may reflect the conjunctive focus of degeneration due to stroke in spatially different sites of cortical and subcortical areas.

The frontal-striatal-thalamic-cortical circuits play an important role in behavioral regulation (Duran et al., 2009), and microstructural change in the anterior limb of the internal capsule was shown to be related to the severity of depressive symptoms in adults with Major Depressive Disorder (MDD) using MRI (Zou et al., 2008). Degeneration in this region may relate to a loss of white matter integrity of these neural circuits (Budde et al., 2007), and this abnormality might trigger the onset of negative mood change. Our finding of a significant negative correlation between the increased ratio of FA values and the change in the depression scale scores 6 months after the stroke might reflect the association between axonal damage of the internal capsule and the negative mood change in stroke patients, and it indicated that recovery from microstructural abnormalities decreased the vulnerability to post-stroke depression, as predicted by elevated depression scale scores.

Our study has some limitations. First, the sample size was not large enough to elucidate the moderate size of difference between groups, and further study with increased numbers of subjects is necessary to draw any definitive conclusions. Second, the stroke patients had predominantly suffered a modest ischemic insult. The extent to which our findings are related to the severity of the ischemic insult was uncertain. Third, patients with significant comprehension deficits were excluded because clinical verbal interviews could not be conducted. Finally, we were not able to control precisely for important variables such as social support, pre-morbid personality, and concurrent and past medication histories. Further analysis, inclusive of consideration of these points, is needed to confirm our present findings.

In conclusion, the present study suggests that FA reduction in the bilateral anterior limbs of the internal capsule is evident in stroke patients. This regional axonal damage should be related to abnormality of neuroanatomical pathways in frontal-subcortical circuits, recent studies of which have highlighted the specific relevance in the pathophysiology of depression due to stroke (Terroni et al., 2011; Paradiso et al., 2013), and it may increase

**Table 4**
Change in psychometric scores and FA values and axial/radial diffusivity over 6 months in patients ($n=18$) and controls ($n=19$).

| | 10–28 Days after stroke | 6 Months after first exam | Paired $t$-test | $p$ |
|---|---|---|---|---|
| *Patients* | | | | |
| Age (years) | 69.2 ± 8.0 | – | – | – |
| Female sex ($n$, %) | 5 (26.3) | – | – | – |
| mRS score | 2.1 ± 0.8 | 1.6 ± 0.6 | $t_{17}=4.12$ | < 0.01** |
| MMSE score | 27.6 ± 3.5 | 29.0 ± 2.1 | $t_{17}=2.08$ | 0.05 |
| SDS score | 26.9 ± 5.6 | 29.2 ± 11.5 | $t_{17}=0.93$ | 0.37 |
| HAM-D score | 3.1 ± 2.7 | 2.8 ± 4.9 | $t_{17}=0.21$ | 0.84 |
| Anterior limb of internal capsule | | | | |
| FA | 0.42 ± 0.06 | 0.44 ± 0.06 | $t_{17}=2.16$ | 0.05 * |
| Axial diffusivity ( × $10^{-3}$) | 4.13 ± 0.37 | 4.29 ± 0.45 | $t_{17}=1.32$ | 0.21 |
| Radial diffusivity ( × $10^{-3}$) | 3.91 ± 0.34 | 4.01 ± 0.37 | $t_{17}=1.38$ | 0.37 |
| *Controls* | | | | |
| Age (years) | 69.1 ± 8.0 | | | |
| Female sex ($n$, %) | 10 (55.5) | | | |
| MMSE score | 29.2 ± 1.2 | 29.7 ± 0.5 | $t_{18}=0.95$ | 0.36 |
| SDS score | 22.1 ± 1.8 | 22.8 ± 2.6 | $t_{18}=1.80$ | 0.10 |
| HAM-D score | 0.4 ± 0.6 | 0.3 ± 0.6 | $t_{18}=1.00$ | 0.33 |
| Anterior limb of internal capsule | | | | |
| FA | 0.49 ± 0.03 | 0.48 ± 0.03 | $t_{18}=0.76$ | 0.46 |
| Axial diffusivity ( × $10^{-3}$) | 4.18 ± 0.66 | 4.30 ± 0.55 | $t_{18}=0.92$ | 0.37 |
| Radial diffusivity ( × $10^{-3}$) | 3.86 ± 0.60 | 4.00 ± 0.46 | $t_{18}=0.75$ | 0.46 |

mRS=Modified Rankin Scale. MMSE=Mini-Mental State Examination. SDS=Zung Self-Rating Depression Scale. HAM-D=Hamilton Rating Scale for Depression.
Data are mean ± S.D.
* $p<0.05$.
** $p<0.01$.

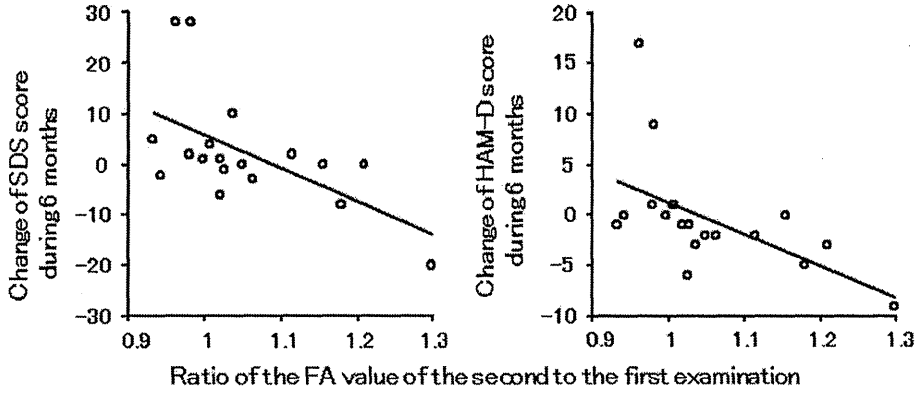


**Fig. 2.** Scatter plots showing the relationship between the ratio of the FA values of the second to the first examination and the change of depression scale scores among patients ($n=18$) Significant correlations were observed between the ratio of the FA values of the second examination to the first examination, and the changes in depression scale scores ($r=-0.59$, $p=0.01$ for the SDS; $r=-0.88$, $p=0.0001$ for the HAM-D).

the risk of post-stroke depression in conjunction with psychosocial factors following stroke. Further, a significant increase of FA in this region was noted 6 months after the stroke, and an association with a reduction in depression scale scores was revealed. Our findings suggested that the promotion of recovery from the axonal damage of stroke patients might prevent the onset of depressive symptoms. Further investigations are needed to clarify whether and how axonal damage of the internal capsule in stroke patients affects the clinical presentation, treatment response and outcome of depression or other psychiatric conditions in stroke survivors.

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Research Article Open Access

# Possible Protective Effect of Regulatory T cells on White Matter Microstructural Abnormalities in Stroke Patients

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## Abstract

**Background:** Despite advances in the understanding of stroke, therapeutic options for stroke are limited. Inflammatory mechanisms activated after brain ischemia are a key target of translational cerebrovascular research. The purpose of the present study was to investigate the existence of microstructure abnormalities in the white matter of stroke patients and their relationship to lymphocyte subsets.

**Methods:** The study included 18 patients with acute ischemic stroke and 22 healthy subjects. Diffusion tensor scans with magnetic resonance imaging were performed. Whole brain voxel-based analysis was used to compare fractional anisotropy (FA) in the stroke and healthy control groups. Blood samples were obtained from all subjects at the initial examination. The lymphocyte subsets in peripheral blood were evaluated with flow cytometric analysis. Helper T cells ($CD3^+$ and $CD4^+$), cytotoxic T cells ($CD3^+$ and $CD8^+$), B cells ($CD19^+$), natural killer cells ($CD16^+$ or $CD56^+$), and regulatory T cells ($T_{regs}$) ($CD4^+$, $CD25^+$, and $FOXP3^+$) were identified.

**Results:** In the voxel-based analysis, FA in the bilateral anterior limbs of the internal capsule was lower in stroke patients than in healthy subjects. These regions exhibited decreased axial diffusivity. The frequency of $T_{regs}$ was lower in patients than in healthy controls. In patients, we found a significant positive relationship between the level of circulating $T_{regs}$ and the FA value in the anterior limb of the internal capsule.

**Conclusions:** Patients exhibited a decreased frequency of circulating $T_{regs}$ and the degree of reduction correlated with the decrease in the FA value in the internal capsule. $T_{regs}$ might attenuate post-stroke white matter tissue damage by limiting the immune response. Our findings demonstrate the need for further study of the role of $T_{regs}$ in the prevention of post-stroke cerebral damage.

**Keywords:** Stroke; Magnetic resonance imaging (MRI); Diffusion tensor imaging (DTI); Fractional anisotropy (FA); Regulatory T lymphocyte ($T_{reg}$)

## Introduction

Stroke is the third leading cause of death and the most frequent cause of permanent disability in adults worldwide [1]. Despite considerable advances in understanding the pathophysiology of cerebral ischemia, therapeutic options for stroke are limited. Inflammatory mechanisms activated after brain ischemia are a key target of current translational cerebrovascular research. Stoke induces a profound local inflammatory response involving various types of immune cells that transmigrate across the activated blood-brain barrier to invade the brain [2].

In the search for ways to prevent cerebral damage due to stroke, several factors related to inflammation have received considerable attention [3-5]. In particular, T lymphocytes are central to the development of a sustained inflammatory response in brain injury after a stroke. T cells are sources of pro-inflammatory cytokines and cytotoxic substances, such as reactive oxygen species, that likely contribute to neuronal death and poor outcome in stroke. However, recent evidence has indicated a novel role of T cells in promoting brain tissue repair and regeneration in the weeks and months after a stroke [6]. The complex role of T lymphocytes in ischemic stroke remains poorly understood. Further research is needed to understand which T-cell subpopulations produce and prevent damage after a stroke.

The primary aim of the present study was to elucidate the microstructural abnormalities in the white matter circuit in stroke patients and determine their relationship to the levels of circulating T lymphocytes. To identify microstructural abnormalities in stroke

patients, diffusion tensor imaging was performed and whole brain voxel-based analysis was used to compare fractional anisotropy (FA) in acute ischemic stroke patients and healthy control subjects. Furthermore, the circulating T-cell subpopulations in stroke patients and healthy subjects were compared, and the association between T-cell subpopulations and white matter microstructural abnormalities in patients was assessed.

## Methods

### Subjects

After providing subjects with a complete description of the study, written informed consent was obtained. The study was approved by the medical ethics committee of the National Cerebral and Cardiovascular Center of Japan. The patients were of Japanese ethnicity and were recruited from the neurology unit of the National Cerebral and Cardiovascular Center hospital. The patients had initially been hospitalized for treatment of acute ischemic stroke.

Stroke was diagnosed by neurologists according to the WHO criteria (1989). After the assessment, a group of psychiatrists and neurologists reviewed the data and reached a consensus regarding the presence or absence of psychiatric disease, including dementia, according to DSM-IV criteria. Patients were included if they met the following criteria: 1) a focal lesion of either the right or left hemisphere on magnetic resonance imaging (MRI); 2) absence of other neurologic, neurotoxic, and metabolic conditions; 3) modest ischemic insult (modified Rankin scale ≤ 4) with absence of a significant verbal comprehension deficit; and 4) occurrence of stroke 10–28 days before the examinations. The exclusion criteria were: 1) transient ischemic attack, cerebral hemorrhage, subdural hematoma, or subarachnoid hemorrhage; 2) history of a central nervous system disease such as tumor, trauma, hydrocephalus, or Parkinson's disease; and 3) pre-stroke history of depression. Eighteen subjects participated in the MRI study and the analysis of lymphocyte subsets in peripheral blood.

Twenty-two healthy control subjects were recruited for this study from the local area by poster advertisement. Subjects were excluded if they had a history or current diagnosis of any DSM-IV axis I or neurological illness. The major characteristics of this cohort are summarized in Table 1.

| Characteristic | Stroke patients (n = 18) | Healthy control subjects (n = 22) | t or $\chi^2$ | p |
|---|---|---|---|---|
| Age (years) | 70.0 ± 6.7 | 67.2 ± 5.5 | t=1.46 | 0.15 |
| Female, n (%) | 4 (22.2) | 8 (36.3) | $\chi^2$=0.94 | 0.33 |
| MMSE score | 28.4 ± 1.9 | 29.3 ± 1.0 | t=1.98 | 0.06 |
| **History of disease, n (%)** | | | | |
| Diabetes mellitus | 5 (27.8) | 2 (9.1) | $\chi^2_1$=2.40 | 0.12 |
| Hyperlipidemia | 5 (27.8) | 1 (4.5) | $\chi^2_1$=4.19 | 0.04* |
| Hypertension | 14 (77.8) | 5 (22.7) | $\chi^2_1$=12.0 | <0.01** |
| **Fazekas DWMH score, n (%)** | | | | |
| 0–2 | 11 (61.1) | 22 (100) | | |
| 3 | 7 (38.9) | 0 (0.0) | $\chi^2$=10.4 | <0.01** |
| **Fazekas PVH score, n (%)** | | | | |
| 0–2 | 13 (72.2) | 22 (100) | | |
| 3 | 5 (27.8) | 0 (0.0) | $\chi^2$=6.98 | <0.01** |
| mRS score | 1.9 ± 0.7 | - | | |
| NIHSS score | 2.8 ± 0.9 | - | | |
| **Anti-coagulant/platelet medication, n (%)** | | | | |
| Warfarin | 3 (16.7) | | | |
| Acetylsalicylic acid | 13 (72.2) | | | |
| Clopidogrel sulfate | 2 (11.1) | | | |
| Cilostazol | 3 (16.7) | | | |
| Number of acute infarcts | 1.2 ± 0.5 | - | | |
| Volume of acute infarcts (mL) | 1.6 ± 0.9 | - | | |
| **Acute infarct location, n (%)** | | | | |
| Basal ganglia | 11 (61.1) | - | | |
| Subcortical white matter | 6 (33.3) | | | |
| Thalamus | 1 (5.6) | - | | |
| **Laterality of acute hemisphere infarcts** | | | | |
| Left hemisphere, n (%) | 9 (50.0) | - | | |

Data are the mean ± SD. * p < 0.05, ** p < 0.01; MMSE: Mini-Mental State Examination; DWMH: Deep White Matter Hyperintensity; PVH: Periventricular Hemorrhage; mRS: Modified Rankin Scale; NIHSS: National Institutes of Health Stroke Scale

**Table 1:** Demographic characteristics of patients and healthy control subjects.

All patients received a neurological examination (modified Rankin Scale (mRS) [7], National Institutes of Health Stroke Scale (NIHSS) [8]) on the day of the MRI scan. Cognitive function was measured with the mini-mental state examination (MMSE) [9] in patients and control subjects. MRIs were conducted for all of the study subjects. The severity of white matter hyper intensity (WMH) was classified using the Fazekas scale, which is a simple visual rating scale used to rate the degree of leukoaraiosis (WMH). It provides an assessment of WMH in the peri-ventricular area (PVH) and in deep white matter (DWMH) on a four-point scale (0–3) [10].

### MRI data acquisition

All MRI examinations were performed using a 3-Tesla whole-body scanner (Signa Excite HD V12M4; GE Healthcare, Milwaukee, WI, USA) with an 8-channel phased-array brain coil. Diffusion tensor images were acquired with a locally modified single-shot echo-planar imaging sequence by using parallel acquisition at a reduction (ASSET) factor of 2 in the axial plane. Imaging parameters were as follows: Repetition Time (TR)=17 seconds; Eco Time (TE)=72 ms; b=0 and

1000 seconds/mm$^2$; acquisition matrix, 128 × 128; field of view (FOV), 256 mm; section thickness, 2.0 mm; no intersection gap; 74 sections. The reconstruction matrix was the same as the acquisition matrix, and 2 mm × 2 mm × 2 mm isotropic voxel data were obtained. A motion probing gradient was applied in 55 directions, and 4144 images were obtained. The acquisition time was 15 min 52 seconds.

To reduce blurring and signal loss arising from field in homogeneity, an automated high-order shimming method based on spiral acquisitions [11] was used before acquiring diffusion tensor imaging scans. FMRIB software (FMRIB Center, Department of Clinical Neurology, University of Oxford, Oxford, England; http://www.fmrib.ox.ac.uk/fsl/) was used to correct for motion and distortion from eddy current and B0 in homogeneity. B0 field mapping data were also acquired with the echo time shift (2.237 msec) method based on two gradient echo sequences.

High-resolution, three-dimensional T1-weighted images were acquired using a spoiled gradient-recalled sequence (TR=12.8 msec; TE=2.6 msec; flip angle=8°; FOV, 256 mm; 188 sections in the sagittal plane; acquisition matrix, 256 × 256; acquired resolution, 1 × 1 × 1 mm). T2-weighted images were obtained using a fast-spin echo (TR=4800 ms; TE=101 ms; echo train length (ETL)=8; FOV=256 mm; 74 slices in the transverse plane; acquisition matrix, 160 × 160; acquired resolution, 1 × 1 × 2 mm).

### Image processing

Fractional anisotropy maps, diffusion weighted images, and three eigen values (λ1, λ2, and λ3) were generated for each individual using FMRIB software. First, brain tissue was extracted using the Brain Extraction Tool. Brain maps for each of the 55 directions were eddy-corrected. Subsequently, FA values were calculated at each voxel using the FSL FMRIB Diffusion Toolbox.

Image preprocessing and statistical analysis were carried out using SPM8 software (Welcome Department of Imaging Neuroscience, London, England). Each subject's echo planar image was spatially normalized to the Montreal Neurological Institute echo planar image template using parameters determined from the normalization of the image with a b value of 0 seconds/mm$^2$. Images were resampled with a final voxel size of 2 × 2 × 2 mm$^3$. Normalized maps were spatially smoothed using an isotropic Gaussian filter (8-mm full-width at half-maximum).

### Voxel-based analysis

Voxel-based analysis was performed using SPM8 software. FA maps of patients and healthy subjects were compared using analysis of covariance (ANCOVA), with age and sex as covariates. Statistical inferences were made with a voxel-level threshold of $p<0.001$, uncorrected, and a minimum cluster size of 100 voxels. The regional FA value was calculated by averaging the FA values for all voxels within the volume of interest (VOI), corresponding to the cluster composed of significant contiguous voxels in the above analysis. The same VOIs were applied to $\lambda_1$ - $\lambda_3$ images, and $\lambda_1$ - $\lambda_3$ values were extracted. Axial ($\lambda_1$) and radial diffusivity ($[\lambda_2 + \lambda_3]/2$) were compared.

### Flow cytometric analysis of lymphocyte subsets in peripheral blood

Blood samples (5 mL) were obtained from all patients and healthy control subjects at the initial examination. The samples were collected into tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's protocol. PBMCs were washed twice with phosphate buffered saline containing 1% fetal calf serum and 2 mM ethylene diamine tetra acetate.

To identify helper T cells ($CD3^+$ and $CD4^+$), cytotoxic T cells ($CD3^+$ and $CD8^+$), B cells ($CD19^+$), and natural killer (NK) cells ($CD16^+$ or $CD56^+$), the PBMCs were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (Beckman Coulter, Orange Country, CA, USA), phycoerythrin-cyanin 5 (PC5)-conjugated anti-human CD4 (Beckman Coulter), phycoerythrin-cyanin 7 (PC7)-conjugated anti-human CD8 (Beckman Coulter), phycoerythrin (PE)-conjugated anti-human CD19 (Beckman Coulter), PC5-conjugated anti-human CD16 (Beckman Coulter), and/or PE-conjugated anti-human CD56 (Beckman Coulter) at 4°C for 20 min. To identify $T_{regs}$ ($CD4^+$, $CD25^+$, and $FOXP3^+$), PBMCs were incubated with FITC-conjugated anti-human CD4 (Beckman Coulter) and PC5-conjugated anti-human CD25 (Beckman Coulter) at 4°C for 20 min. After surface staining, PBMCs were fixed, permeabilized, and stained with PE-conjugated anti-human FOXP3 (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. As negative controls, fluorochrome-conjugated non-specific isotype-matched antibodies (Beckman Coulter) were used. Stained cells were analyzed using a FC500 cytometer and CXP software (Beckman Coulter). The percentage of cells stained with a particular antibody was reported after subtracting the percentage of cells stained with the relevant negative isotype control antibodies.

### Statistical analysis

Group differences in the demographic characteristics of patients and healthy controls were examined with an unpaired t-test and Pearson's $\chi^2$ test. To examine group differences in FA values and axial/radial diffusivity in VOIs from the voxel-based analysis, we performed ANCOVA with age and sex as covariates.

We also used ANCOVA with age and sex as covariates to examine differences in the percentage of helper T cells, cytotoxic T cells, regulatory T cells, B cells, and NK cells between patients and healthy controls. For cells that showed significant differences between groups, the correlation between the FA values and the percentage of cells was examined using Spearman's correlation analysis.

All statistical tests were two-tailed and reported at $p<0.05$. The Bonferroni correction was applied to avoid type I errors due to the multiplicity of statistical analyses. Statistical analysis of the data was performed using SPSS for Windows 19.0 (IBM Japan Inc., Tokyo, Japan).

## Results

### Demographic and clinical data

Table 1 summarizes the demographic and clinical characteristics of the study subjects. Patients did not differ significantly from healthy control subjects in age, sex, or MMSE scores. The occurrence of hyperlipidemia and hypertension was significantly higher in patients than in healthy controls. There were no healthy control subjects with Fazekas scores higher than 3. Table 1 also shows the mRS and NIHSS scores, treatment with anti-coagulant/platelet medication, and the location and volume of the infarct. Patients exhibited some disability

from stroke at the time of the examination. All patients took anti-coagulant and/or anti-platelet medicine. Infarction occurred in the basal ganglia (61.1%), sub-cortical white matter (33.3%), and thalamus (5.6%). There was no significant laterality of hemisphere infarcts. Representative MR images of patients and controls are shown in Figure 1.

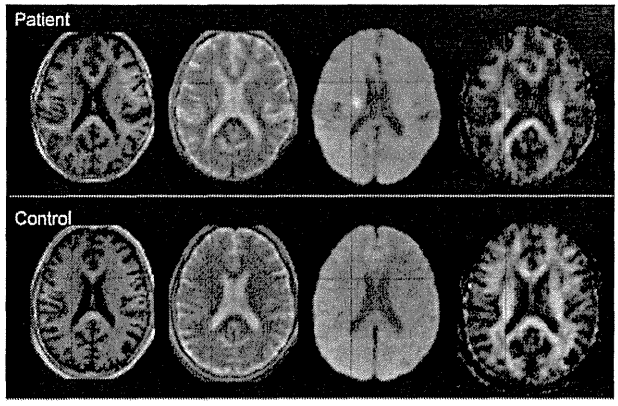


**Figure 1: Representative MR images for patients and controls** T1-weighted, T2-weighted, diffusion weighted, and fractional anisotropic (FA) images are shown, respectively, from left to right. All figures were spatially normalized to the image template in SPM8, and the axial slices are shown (z=18). The vertical and horizontal lines in the images represent the coordinates of x=-26 and y=12, respectively.

## FA values in patient and control groups

In the voxel-based analysis of FA values, the white matter FA values in the left and right anterior limbs of the internal capsule differed in the patient and healthy control groups [left anterior limb of internal capsule: (x, y, z)=(-26,12,18), cluster voxel size=831, T=5.20; right anterior limb of internal capsule: (x,y,z)=(26,16,4), cluster voxel size=487, T=5.24] (Figure 2A). Figure 2B shows scatter plots of the FA values of the anterior limb of the internal capsule. Table 2 shows the FA values and radial/axial diffusivity in the affected regions. Decreased axial diffusivity, but no change in radial diffusivity, was observed in the affected regions.

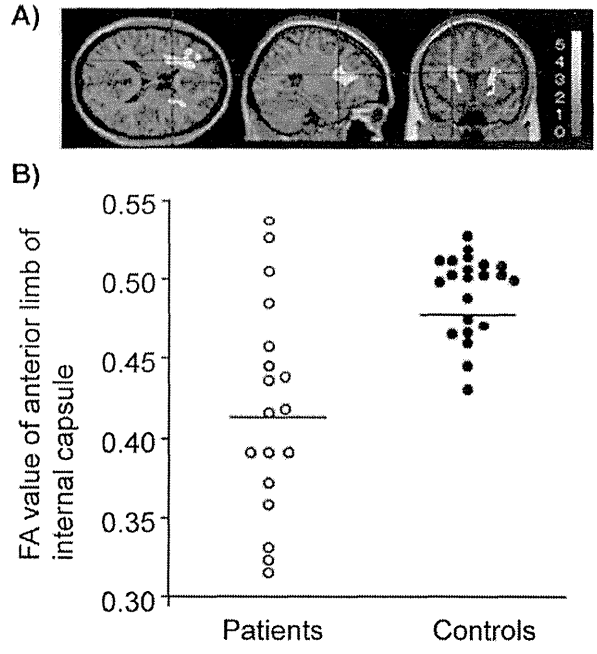


**Figure 2: White matter fractional anisotropy (FA) in stroke patients and control subjects. (A)** Voxel-based analysis was performed using SPM8 software. FA maps of patients (n=18) and healthy subjects (n=22) were compared using analysis of covariance (ANCOVA), with age and sex as covariates. Statistical inferences were made with a voxel-level threshold of $p<0.001$, uncorrected, and a minimum cluster size of 100 voxels. Statistical parametric mapping projections were superimposed on a representative magnetic resonance image (x=-26, y=12, z=18). FA in the right and left anterior limbs of the internal capsule was reduced in stroke patients. **(B)** Scatter plots of FA values in the regions of FA reduction in stroke patients. The regional FA value was calculated by averaging the FA values for all voxels within the volume of interest corresponding to the cluster composed of significant contiguous voxels in the preceding analysis. In the bilateral anterior limbs of the internal capsule, the FA values of stroke patients were lower than those of healthy subjects ($p<0.01$).

| | | | Analysis of covariance † | |
|---|---|---|---|---|
| **FA and axial/radial diffusivity** | **Stroke patients (n=18)** | **Healthy controls (n=22)** | **F (1, 36)** | **p** |
| **Left anterior limb of internal capsule** | | | | |
| FA | 0.41 ± 0.08 | 0.48 ± 0.03 | 16.4 | <0.001** |
| Axial diffusivity ($\times 10^{-3}$) | 4.16 ± 0.32 | 4.37 ± 0.30 | 4.24 | 0.05 * |
| Radial diffusivity ($\times 10^{-3}$) | 3.96 ± 0.30 | 4.04 ± 0.29 | 0.48 | 0.49 |
| **Right anterior limb of internal capsule** | | | | |
| FA | 0.43 ± 0.06 | 0.50 ± 0.03 | 23.0 | <0.001** |

| | | | | |
|---|---|---|---|---|
| Axial diffusivity ($\times 10^{-3}$) | 4.14 ± 0.33 | 4.35 ± 0.30 | 4.03 | 0.05 |
| Radial diffusivity ($\times 10^{-3}$) | 3.93 ± 0.31 | 4.01 ± 0.30 | 0.46 | 0.50 |
| **Bilateral anterior limbs of internal capsule** | | | | |
| FA | 0.42 ± 0.07 | 0.49 ± 0.03 | 20.6 | <0.001** |
| Axial diffusivity ($\times 10^{-3}$) | 4.15 ± 0.32 | 4.36 ± 0.30 | 4.15 | 0.05 * |
| Radial diffusivity ($\times 10^{-3}$) | 3.95 ± 0.30 | 4.02 ± 0.29 | 0.47 | 0.50 |
| † Age and sex were entered as covariates; Data are the mean ± SD. * $p < 0.05$, ** $p < 0.01$; FA: Fractional Anisotropy; VOI: Volume of Interest | | | | |

**Table 2:** FA values and axial/radial diffusivity in the VOI in patients and healthy control subjects.

| | | | Analysis of covariance † | |
|---|---|---|---|---|
| | **Stroke patients (n=18)** | **Healthy controls (n=22)** | **F (1, 36)** | **p** |
| Helper T lymphocyte (% $CD3^+$) | 67.2 ± 15.1 | 61.5 ± 12.0 | 1.00 | 0.32 |
| Cytotoxic T lymphocyte (% $CD3^+$) | 27.9 ± 13.5 | 33.2 ± 10.6 | 0.92 | 0.35 |
| B lymphocyte (% lymphocyte) | 17.7 ± 8.3 | 12.2 ± 7.8 | 5.42 | 0.03 |
| NK cell (% lymphocyte) | 21.6 ± 11.3 | 27.5 ± 10.4 | 1.78 | 0.19 |
| Regulatory T lymphocyte (% $CD4^+$) | 2.1 ± 1.6 | 3.8 ± 2.3 | 7.89 | 0.008 * |
| † Age and sex were entered as covariates; Data are the mean ± SD. * Significant after correction for multiple statistical tests to avoid type I errors ($p < 0.01$ [0.05/5]); NK: Natural Killer | | | | |

**Table 3:** Percentages of circulating lymphocytes in patients and healthy control subjects.

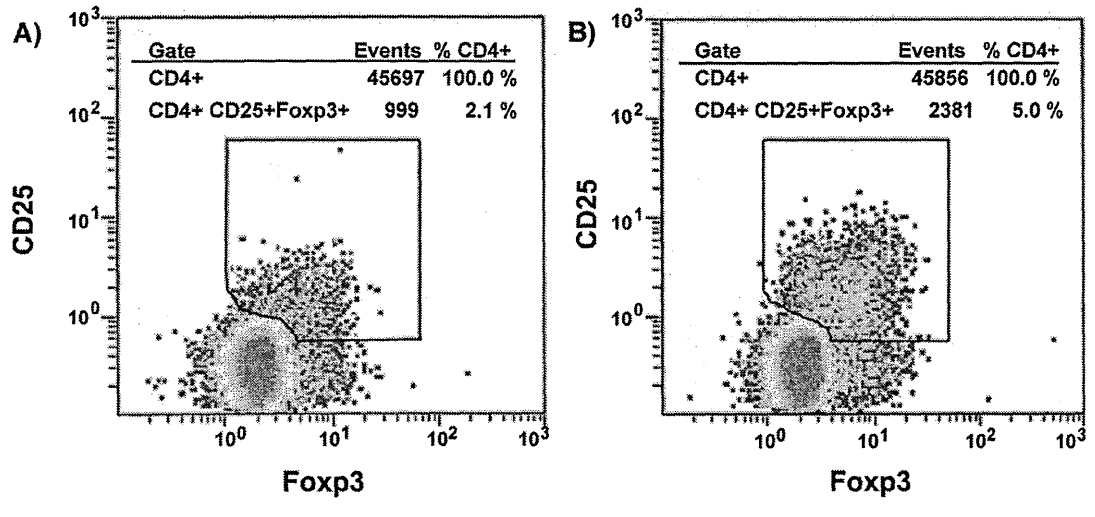


**Figure 3: Flow cytometry analysis of the frequency of CD4+CD25+Foxp3+ cells within the CD4+ T-cell population.** Representative plots are shown. The framed dots represent $CD4^+CD25^+Foxp3^+$ T cells ($T_{regs}$). The percentage of $T_{regs}$ in patients (A) and controls (B) was 2.1% and 5.0%, respectively.

### Lymphocyte subsets and their correlation with FA values in patients

The percentage of Tregs was significantly lower in patients than in healthy controls (Table 3). For patients and controls, representative flow cytometry analysis plots of the frequency of Tregs within the CD4+ T cell population are shown in Figure 3. Scatter plots depicting the percentage of Tregs in patients and controls are shown in Figure 4A. In patients, we found a significant positive relationship between the level of circulating Tregs and the FA value in the anterior limb of the internal capsule (r=0.50, p=0.04) (Figure 4B).

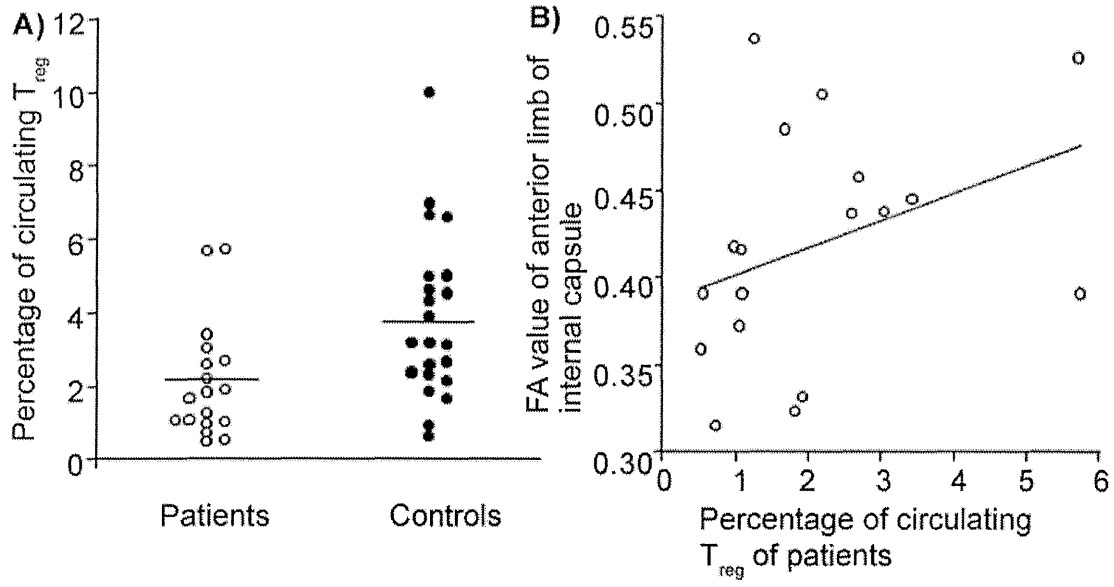


**Figure 4: $T_{reg}$ percentages in patients and controls and the relationship between the percentage of circulating Tregs and the FA value of the anterior limb of the internal capsule in patients. (A)** The percentage of circulating Tregs differed significantly in the stoke patient and healthy control groups ($F1,36 = 7.89$, $p = 0.008$). **(B)** In stroke patients, a significant correlation was observed between the percentage of circulating $T_{regs}$ and the FA value (r=0.50, p=0.04).

## Discussion

Our findings showed that stroke patients had lower FA in the bilateral anterior limbs of the internal capsule when compared to healthy control subjects. The reduced FA in stroke patients was associated with decreased axial diffusivity. Axonal damage leads to a marked decrease in axial diffusivity, and demyelination leads to an increase in radial diffusivity [12]. Therefore, our finding of the reduced FA was a result not of demyelination but of a gross reduction in axonal number and/or size, possibly reflecting Wallerian degeneration secondary to neuronal loss due to stroke [13]. From an anatomical perspective, the anterior limb of the internal capsule represents the intercept point in the course of the frontal-subcortical circuits [14]; it has extensive connectivity with the cortical and subcortical areas. The reduced FA in the anterior limb of the internal capsule may reflect the conjunctive focus of degeneration due to stroke in spatially different sites of the cortical and subcortical areas [15].

Our results demonstrated that the percentage of circulating $T_{regs}$ was lower in stroke patients than in healthy controls. The degree of reduction correlated with the decrease in the FA value in the internal capsule. This may indicate that a decrease in $T_{regs}$ is associated with axonal damage in the internal capsule in stroke patients. After an ischemic stroke, activated T lymphocytes infiltrate the brain and function as a source of pro-inflammatory cytokines and cytotoxic substances [16-18]. However, not all T-cell subtypes are detrimental to acute stroke outcome, and there is evidence that T cells promote brain tissue repair and regeneration. $T_{regs}$ are an important T-cell subtype; they support brain tissue repair and regeneration [19]. Infarct volume and neuronal dysfunction increased when mice were treated with an anti-CD25 monoclonal antibody to neutralize $T_{regs}$ [20]. The ability of $T_{regs}$ to protect the brain and improve stroke outcome was confirmed by Li et al. [21] who studied the effects of post-stroke $T_{reg}$ therapy.

Although the interaction between the brain and the immune system subsequent to ischemic stroke has only recently been documented, the functional role of $T_{regs}$ in other pathological conditions has been studied extensively. Most studies have ascribed the protective effect of $T_{regs}$ to the alleviation of an excessive inflammatory response. It is reasonable to attribute the neuroprotective effect of $T_{regs}$ in stroke to a similar mechanism [22].

$T_{regs}$ limit the immune response by releasing IL-10, an anti-inflammatory cytokine [23]. In experimental brain ischemia with anti-CD25 monoclonal antibody-mediated depletion of $T_{regs}$, the addition of exogenous IL-10 reduced infarct volume [20]. $T_{regs}$ also limit the immune response by releasing transforming growth factor-β, which may be required for neurogenesis [24]. In addition, $T_{regs}$ conferred protection against stroke by blunting the increase in metalloproteinase-9 (MMP-9) [21]. Stroke-induced MMP-9 production resulting from neutrophil infiltration contributed to the breakdown of the blood-brain barrier and promoted leukocyte

infiltration and brain damage [25], whereas $T_{reg}$ adoptive treatment inhibited MMP-9 production in the blood and the brain after ischemia [21].

The reason circulating $T_{reg}$ levels were lower in stroke patients are not clear. People with lower levels of circulating $T_{regs}$ may be more likely to develop stroke and experience severe axonal damage because the activity of their inflammatory system is excessive. Alternatively, the reduction in circulating $T_{regs}$ after stroke may reflect the migration of $T_{regs}$ to brain tissue for the repair of cerebral neuronal injuries. Our findings are based on cross-sectional data without immunohistochemical analysis, which limits our ability to determine which explanation is correct. However, our results are consistent with previous reports describing the brain-protective and outcome-improving effects of $T_{regs}$. Our findings raise the possibility that stroke outcome can be improved by targeting $T_{regs}$ to protect the brain from tissue damage after stroke.

Whereas Liesz et al. [20] showed a protective effect of $T_{regs}$ in stroke, Ren et al. [26] found that $T_{regs}$ had no effect, and Kleinschnitz et al. [27] showed that $T_{regs}$ exacerbated brain injury early after transient ischemia. However, the animal models used in the studies differed in several aspects, including the duration of ischemia and the methods for Treg depletion. In our study, the stroke patients predominantly suffered a modest ischemic insult, and their circulating lymphocytes were studied after 10–28 days. Differences in the severity and stage of ischemic insult may account for the conflicting results.

Our study has a few limitations. First, patients with significant comprehension deficits were excluded because clinical verbal interviews could not be conducted. Second, because our study was an *in vivo* human brain study, immunohistochemical analysis of brain tissue was not possible. Third, all of the patients took anti-coagulant/platelet medicine. Of note, 13 patients took acetylsalicylic acid, which has an anti-inflammatory effect and may have affected our results. However, the extent to which the results were affected by medication remains uncertain. Finally, we did not investigate the other functionally unique $CD4^+$ T-cell subsets (Th1, Th2, and Th17) in this study. A previous study reported that $T_{regs}$/Th17 cells were imbalanced in patients with cerebral infarction: the Th17 frequency increased, and the $T_{reg}$ frequency decreased, relative to the balance in control subjects [28]. Th17 cells orchestrate tissue inflammation, whereas $T_{regs}$ limit the immune response and prevent tissue damage. An imbalance between $T_{regs}$ and Th17 cells in stroke patients may contribute to tissue damage [28]. Further analysis, taking these points into consideration, is needed to confirm our present findings.

In conclusion, the present study suggests that FA is reduced in the bilateral anterior limbs of the internal capsule in stroke patients. In addition, the percentage of circulating $T_{regs}$ was reduced in stroke patients. The degree of reduction correlated with the decrease in the FA value in the internal capsule. $T_{regs}$ might alleviate post-stroke white matter tissue damage by limiting the immune response. Further study of the role of $T_{regs}$ in preventing post-stroke cerebral damage is needed.

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ORIGINAL ARTICLE

# Microstructural abnormality in white matter, regulatory T lymphocytes, and depressive symptoms after stroke

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***Key words:*** *diffusion tensor imaging, fractional anisotropy (FA), magnetic resonance imaging (MRI), post-stroke depression, regulatory T lymphocytes ($T_{reg}$), stroke.*

## Abstract

**Background:** The purpose of the present study was to investigate the existence of microstructure abnormalities in the white matter circuit in stroke patients and its relationship to depressive episodes. To target the prevention of depression, we also investigated the relationship between lymphocyte subsets and cerebral abnormalities in patients.

**Methods:** Participants included 18 patients with acute ischemic stroke and 22 healthy control subjects. Diffusion tensor imaging was performed. Whole-brain voxel-based analysis was used to compare fractional anisotropy (FA) between groups. Blood samples were obtained, and the lymphocyte subsets were evaluated using flow cytometry. Follow-up examinations were conducted on 12 patients at 6 months.

**Results:** FA was decreased in the bilateral anterior limb of the internal capsule in stroke patients. At the 6-month follow-up examination, there was a significant increase in FA, which was associated with a lower depression scale score. Patients showed a decreased percentage of circulated regulatory T lymphocytes, and the degree of reduction was related to the decrease in the FA value in the internal capsule.

**Conclusions:** FA reductions in the anterior limb of the internal capsule cause abnormality in the frontal-subcortical circuits and confer a biological vulnerability, which in combination with environmental stressors results in the onset of depression. Our findings also demonstrated the possibility of preventing post-stroke depression by targeting the role of regulatory T lymphocytes in brain tissue repair and regeneration after stroke.

## INTRODUCTION

Stroke is the third leading cause of death and the most frequent cause of permanent disability in adults worldwide.[1] Depression is common and serious emotional symptom following stroke and is associated with excess disability, cognitive impairment, and mortality.[2] Despite considerable advances in understanding the pathophysiology of cerebral ischemia, therapeutic options for stroke and its related emotional symptoms are still limited. Inflammatory mechanisms activated after brain ischemia represent a key target of current translational cerebrovascular research. Stroke induces profound local inflammatory response involving various types of immune cells that transmigrate across the activated blood–brain barrier to invade the brain.[3]

In attempts to target the prevention of cerebral damage due to stroke, several factors related to inflammation have received considerable attention.[4–6] T lymphocytes are especially central to the development of a sustained inflammatory response in brain injury after a stroke. T cells are sources of pro-inflammatory cytokines and cytotoxic substances, such as reactive oxygen species, in the brain after a stroke; these likely contribute to neuronal death and poor outcomes. However, recent evidence has

indicated a novel role of T cells in promoting brain tissue repair and regeneration in the weeks and months after a stroke.[7] The role of T lymphocytes in ischemic stroke is complex and remains poorly understood. More research is needed to gain a greater understanding of which T-cell subpopulations produce and prevent damage after a stroke.

The primary aim of the present study was to elucidate the microstructural abnormalities in the white matter circuit in stroke patients, as well as their relationship with depressive symptoms after a stroke. When abnormalities were found and a statistical association with post-stroke depressive symptoms was demonstrated, we investigated their relationship with circulating T lymphocytes. For identification of the microstructural abnormalities in stroke patients, diffusion tensor imaging was performed, and whole-brain voxel-based analysis was used to compare fractional anisotropy (FA) between acute ischemic stroke patients and healthy control subjects. Furthermore, we examined which circulating T-cell subpopulations showed differences in stroke patients when compared to healthy subjects and how such T-cell subpopulations are associated with microstructural abnormalities in the white matter of patients.

## METHODS

### Subjects

After the study was described to subjects, written informed consent was obtained. The study was approved by the medical ethics committee of the National Cerebral and Cardiovascular Center of Japan. The patients, all of whom were of Japanese ethnicity, were recruited from the neurology unit of the National Cerebral and Cardiovascular Center Hospital. These patients had initially been hospitalized for treatment of acute ischemic stroke.

Stroke was diagnosed by neurologists according to the World Health Organization criteria (1989). After the assessment, a group of psychiatrists and neurologists reviewed the data and reached a consensus regarding the presence or absence of psychiatric disease, including dementia according to the Diagnostic and Statistical Manual, 4th edition criteria. Patients were included if they met the following criteria: (i) a focal lesion of either the right or left hemisphere on magnetic resonance imaging (MRI); (ii) absence of other neurologic, neurotoxic, or metabolic conditions; (iii) modest ischemic insult (modified Rankin scale ≤4) with absence of a significant verbal comprehension deficit; and (iv) occurrence of stroke 10–28 days before the examinations. Exclusion criteria included the following: (i) transient ischemic attack, cerebral haemorrhage, subdural haematoma, or subarachnoid haemorrhage; (ii) history of a central nervous system disease such as tumour, trauma, hydrocephalus or Parkinson's disease; and (iii) pre-stroke history of depression. Eighteen subjects met the criteria and participated in this study.

Twenty-two healthy control subjects were recruited locally for this study based on their response to a poster seeking subjects. Exclusion criteria for healthy subjects were a history or present diagnosis of any Diagnostic and Statistical Manual, 4th edition axis I or neurological illness. Major characteristics of this cohort are summarized in Table 1.

All patients were subjected to a neurological examination using the modified Rankin scale (mRS) and the National Institutes of Health Stroke Scale (NIHSS) on the day of the MRI scan.[8,9] A quantitative measurement of cognitive function, the Mini-Mental State Examination (MMSE), and of depressive symptoms, the Hamilton Rating Scale for Depression (HAM-D), was carried out in patients and control subjects. MRI were conducted for all study subjects.[10,11]

At 6 months, follow-up MRI were conducted for 12 of the 18 patients. There were no changes in medication use between baseline and follow-up, and no patients were on antidepressant treatment during the examinations. All patients were subjected to a series of quantitative measurements of depressive symptoms (HAM-D), cognitive function (MMSE), and neurological examination (mRS, NIHSS) on the day of the follow-up MRI scan.[8–11]

### Data acquisition of MRI

All MRI examinations were performed with a 3-Tesla whole-body scanner (Signa Excite HD V12M4; GE Healthcare, Milwaukee, WI, USA) with an eight-channel phased-array brain coil. Diffusion tensor images were acquired with a locally modified single-shot echo-planar imaging sequence that used parallel acquisition at a reduction factor of 2 in the axial plane. Imaging parameters were as follows: repetition time = 17 s; echo time = 72 ms; b value = 0, 1000 $mm^2/s$, acquisition matrix, 128 × 128; field of view, 256 mm; section thickness, 2.0 mm; no intersection gap; 74 sections. The reconstruction matrix was the same as

**Table 1** Demographic characteristics of patients and healthy control subjects

| Characteristic | Stroke patients ($n$ = 18) | Healthy control subjects ($n$ = 22) | $t$ or $\chi^2$ | $P$-value |
|---|---|---|---|---|
| Age (years) | 70.0 ± 6.7 | 67.2 ± 5.5 | $t$ = 1.46 | 0.15 |
| Female sex ($n$) | 4 (22.2%) | 8 (36.3%) | $\chi^2$ = 0.94 | 0.33 |
| MMSE score | 28.4 ± 1.9 | 29.3 ± 1.0 | $t$ = 1.98 | 0.06 |
| HAM-D score | 2.4 ± 2.4 | 1.0 ± 1.5 | $t$ = 2.20 | 0.03* |
| History of disease ($n$) | | | | |
| Diabetes mellitus | 5 (27.8%) | 2 (9.1%) | $\chi^2_1$ = 2.40 | 0.12 |
| Hyperlipidaemia | 5 (27.8%) | 1 (4.5%) | $\chi^2_1$ = 4.19 | 0.04* |
| Hypertension | 14 (77.8%) | 5 (22.7%) | $\chi^2_1$ = 12.0 | <0.01** |
| mRS score | 1.9 ± 0.7 | — | | |
| NIHSS score | 2.8 ± 0.9 | — | | |
| Anticoagulant or anti-platelet medication ($n$) | | | | |
| Warfarin | 3 (16.7%) | | | |
| Acetylsalicylic acid | 13 (72.2%) | | | |
| Clopidogrel sulfate | 2 (11.1%) | | | |
| Cilostazol | 3 (16.7%) | | | |
| Acute infarcts | 1.2 ± 0.5 | — | | |
| Volume of acute infarcts (mL) | 1.6 ± 0.9 | — | | |
| Location of acute infarcts ($n$) | | | | |
| Basal ganglia | 11 (61.1%) | — | 0.611 | |
| Subcortical white matter | 6 (33.3%) | | | |
| Thalamus | 1 (5.6%) | — | 0.056 | |
| Laterality of hemisphere infarcts | | | | |
| Left hemisphere ($n$) | 9 (50.0%) | — | | |

Data are mean ± SD. *$P$ < 0.05, **$P$ < 0.01.
HAM-D, Hamilton Rating Scale for Depression; MMSE, Mini-Mental State Examination; mRS, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale.

the acquisition matrix, and 2 × 2 × 2 mm isotropic voxel data were obtained. Motion-probing gradient was applied in 55 directions, the number of images was 4144, and the acquisition time was 15 min 52 s.

To reduce blurring and signal loss arising from field inhomogeneity, we used an automated high-order shimming method based on spiral acquisitions before acquiring diffusion tensor imaging scans.[12] To correct for motion and distortion from eddy current and $B_0$ inhomogeneity, FMRIB software (FMRIB Center, Department of Clinical Neurology, University of Oxford, Oxford, UK; http://www.fmrib.ox.ac.uk/fsl/fslwiki/) was used. $B_0$ field mapping data were also acquired with the echo time shift (2.237 ms) method based on two gradient echo sequences.

High-resolution 3-D, $T_1$-weighted images were acquired with a spoiled gradient-recalled sequence (repetition time = 12.8 ms, echo time = 2.6 ms, flip angle = 8, field of view, 256 mm; 188 sections in the sagittal plane; acquisition matrix, 256 × 256; acquired resolution, 1 × 1 × 1 mm). $T_2$-weighted images were obtained with a fast-spin echo (repetition time = 4800 ms; echo time = 101 ms; echo train length = 8; field of view = 256 mm; 74 slices in the transverse plane; acquisition matrix, 160 × 160, acquired resolution, 1 × 1 × 2 mm).

### Image processing

Fractional anisotropy (FA) maps and three eigenvalues (λ1, λ2, and λ3) were generated from each individual with FMRIB software. First, brain tissue was extracted using the Brain Extraction Tool. Brain maps for each of the 55 directions were eddy corrected, subsequent to which FA values were calculated at each voxel with the FSL FMRIB Diffusion Toolbox.

Image pre-processing and statistical analysis were carried out using SPM8 software (Wellcome Department of Imaging Neuroscience, London, UK). Each subject's echo planar image was spatially normalized to the Montreal Neurological Institute echo planar image template using parameters determined from the normalization of the image with a b value of 0 $mm^2/s$ and the echo planar image template in SPM8. Images were resampled with a final voxel size of 2 × 2 × 2 $mm^3$. Normalized maps were spatially smoothed using an isotropic Gaussian filter (8-mm full-width at half-maximum).

### Voxel-based analysis

Voxel-based analysis was performed using SPM8 software. FA maps were compared between patients and healthy subjects with ANCOVA, with age and sex as covariates of no interest. Statistical inferences were made with a voxel-level threshold of $P < 0.001$, uncorrected, with a minimum cluster size of 100 voxels. The regional FA value was calculated by averaging the FA values for all voxels within the volume of interest corresponding to the cluster composed of significant contiguous voxels. The same volumes of interest were applied to $\lambda_1$–$\lambda_3$ images, and $\lambda_1$–$\lambda_3$ values were extracted. Axial ($\lambda_1$) and radial diffusivity ($(\lambda_2 + \lambda_3)/2$) were compared.

### Flow cytometric analysis of lymphocyte subsets in peripheral blood

Blood samples (5 mL) were obtained from all of the patients and healthy control subjects at the initial examination. The samples were collected into tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's protocol. PBMC were washed twice with phosphate-buffered saline containing 1% foetal calf serum and 2-mM EDTA.

To identify helper T cells (CD3+ & CD4+), cytotoxic T cells (CD3+ & CD8+), B cells (CD19+) and natural killer cells (CD16+ or CD56+), we incubated the PBMC with fluorescein isothiocyanate-conjugated anti-human CD3 (Beckman Coulter, Orange Country, CA, USA), phycoerythrin-cyanin (PC)5-conjugated anti-human CD4 (Beckman Coulter), PC7-conjugated anti-human CD8 (Beckman Coulter), phycoerythrin-conjugated anti-human CD19 (Beckman Coulter), PC5-conjugated anti-human CD16 (Beckman Coulter), and/or phycoerythrin-conjugated anti-human CD56 (Beckman Coulter) at 4°C for 20 min. To identify regulatory T lymphocytes ($T_{reg}$) (CD4+, CD25+ & FOXP3+), we incubated the PBMC with fluorescein isothiocyanate-conjugated anti-human CD4 (Beckman Coulter) and PC5-conjugated anti-human CD25 (Beckman Coulter) at 4°C for 20 min. After surface staining, PBMC were fixed, followed by permeabilization and staining with phycoerythrin-conjugated anti-human FOXP3 (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. As negative controls, fluorochrome-conjugated non-specific isotype-matched antibodies (Beckman Coulter) were used. Stained cells were analyzed using a FC500 cytometer and CXP software (Beckman Coulter). Percentages of cells stained with a particular antibody are reported after subtraction of the percentage of cells stained with the relevant negative isotype control antibodies.

### Statistical analysis

Group differences in demographic characteristics between patients and healthy controls were examined by unpaired *t*-test and Pearson's $\chi^2$ test. To examine the group differences in FA values and axial/radial diffusivity in volume of interest shown in the voxel-based analysis, we performed ANCOVA with age and sex as covariates.

Paired *t*-tests were performed 6 months after the initial examinations to determine changes in patients' mRS, NIHSS, MMSE, and HAM-D scores and FA values. We performed Pearson's correlation analysis to examine the relationship between FA values and depressive symptoms at the first assessment and at the assessment performed 6 months later. To examine the relationship between the change in depression scale scores and the ratio of the FA values (FA values at second vs initial examination) in patients, we performed Pearson's correlation analysis.

To examine whether the ratio of the FA values was related to the change in depression scale scores (HAM-D scores at second examination minus initial examination), we performed multiple regression analysis after adjustment for age and gender. The change in depression scale scores was the dependent variable, and the ratio of the FA values was the independent variable.

Additionally, we performed ANCOVA with age and sex as covariates to examine the differences in the numbers of helper T cells, cytotoxic T cells, regulatory T cells, B cells, and natural killer cells between patients and healthy control subjects. For the cells showing significant differences between groups, we examined the correlation between FA values and cell number by Spearman's correlation analysis. To determine whether FA values were related to cell number, we performed multiple regression analysis with the FA values as dependent variable and cell number as independent variables, after adjustment for age and gender.