Abstract: The 677 C to T transition in the MTHFR gene is a genetic determinant for hyperhomocysteineemia. We investigated whether this polymorphism modulates gray matter (GM) structural covariance networks independently of white-matter integrity in patients with Alzheimer’s disease (AD). GM structural covariance networks were constructed by 3D T1-magnetic resonance imaging and seed-based analysis. The patients were divided into two genotype groups: C homozygotes (n = 73) and T carriers (n = 62). Using diffusion tensor imaging and white-matter parcellation, 11 fiber bundle integrities were compared between the two genotype groups. Cognitive test scores were the major outcome factors. The T carriers had higher homocysteine levels, lower posterior cingulate cortex GM volume, and more clusters in the dorsal medial lobe subsystem showing stronger covariance strength. Both posterior cingulate cortex seed and interconnected peak cluster volumes predicted cognitive test scores, especially in the T carriers. There were no between-group differences in
fiber tract diffusion parameters. The MTHFR 677T polymorphism modulates posterior cingulate cortex-anchored structural covariance strength independently of white matter integrities. Hum Brain Mapp 38:3039–3051, 2017. © 2017 Wiley Periodicals, Inc.

Key words: Alzheimer’s disease; genetic effect; anatomical structural covariance; default-mode network; posterior cingulate cortex

INTRODUCTION

Both cross-sectional and longitudinal studies have reported that deep and periventricular white matter hyperintensities (WMHs) are of clinical significance in modulating symptoms in patients with Alzheimer’s disease (AD) (Chang et al., 2012; Huang et al., 2010; Tu et al., 2010). In AD, the presence of WMHs is related to breakdown of the blood–brain barrier (de Lau et al., 2010), impaired cerebral autoregulation (Hoth et al., 2008), vasculopathies (Huang et al., 2015), and hyperhomocysteinemia (Huang et al., 2010, 2013). In humans, the nonsynonymous polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene at position 677 may result in reduced MTHFR enzyme activity. The common 677 C to T transition (rs1801133) in the MTHFR gene represents a well-known genetic determinant for hyperhomocysteinemia, and higher levels of homocysteine have been reported in the T variant (Jacques et al., 1996).

Genetic influences on co-morbidities are well known, and associations between the C677T T/T genotype and the risk of uremia (Hishida et al., 2013), and the 677T variant with a reduced estimated glomerular filtration rate (eGFR) in hypertensive Chinese males (Dong et al., 2012) have been reported. In addition, the Hisayama study demonstrated that hyperhomocysteinemia in the general population represents a significant risk factor for the development of chronic kidney disease (Ninomiya et al., 2004). Uremia and a lower eGFR have been reported to affect the integrity of the brain. However, whether the effect of the 677T polymorphism in causing WMHs in patients with AD is confounded by serum homocysteine level, eGFR status or is itself significant for WMHs has yet to be elucidated. In addition, how these factors interact with each other to affect the gray matter (GM) network or WMH load in patients with AD remains unclear.

The concept of structural covariance networks (SCNs) is supported by recent research in that highly related regions may show covariance in morphometric characteristics. SCN patterns reflect both structural and functional connectivity (Alexander-Bloch et al., 2013). Genetic variations, developmental, degenerative, and disease processes each carry different biological weighting which determines the SCN pattern (Lin et al., 2016). Three SCNs have been reported to be relevant to the disease process of AD, the so-called default mode network (DMN) (Greicius et al., 2004; Greicius et al., 2009; Seeley et al., 2007), salience network (Seeley et al., 2007), and executive control network (Agosta et al., 2009; Filippi et al., 2013). A recent report suggested that the DMN may be composed of multiple, spatially dissociated but interactive components, of which two subsystems are particularly relevant: the “medial temporal lobe subsystem,” and the “dorsal medial prefrontal cortex subsystem” (or the midline core subsystem) (Andrews-Hanna et al., 2010).

The potential mechanisms of genetic-based neurobiology are under intensive investigation. Research on the MTHFR C677T functional polymorphism with regards to changes in serum biomarker levels, SCN patterns, WMHs, and relationships with cognitive measurements may reveal whether this genetic variation is of clinical relevance in AD. In this study, we hypothesized that the MTHFR C677T functional polymorphism may modulate selective SCN patterns in patients with AD, and that network alterations may be independent of WMHs.

MATERIALS AND METHODS

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Chang Gung Memorial Hospital. The study participants were treated at the Cognition and Aging Center, Department of General Neurology, Kaohsiung Chang Gung Memorial Hospital. A total of 135 subjects (65 males and 70 females) were included after the consensus of a panel composed of neurologists, neuropsychologists, neuroradiologists, and experts in nuclear medicine (Huang et al., 2015). AD was diagnosed according to the International Working Group criteria (Dubois et al., 2010). All the patients had a clinical dementia rating score of 0.5 or 1, and all were in a stable condition under acetylcholine esterase inhibitor treatment from the time of diagnosis. The exclusion criteria were a past history of clinical stroke, potential cardiovascular diseases such as acute

Abbreviations

AD Alzheimer’s disease
ApoE apolipoprotein E
CASI cognitive ability-screening instrument
DMN default mode network
eGFR estimated glomerular filtration rate
GM gray matter
MMSE Mini-Mental State Examination
MTHFR methylenetetrahydrofolate reductase
SCNs structural covariance networks
WMHs white matter hyperintensities
WM white matter
Clinical and Neurobehavioral Assessments

Demographic data of all patients were recorded after enrolment. A trained neuropsychologist administered the neurobehavioral tests. The total scores of the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) and cognitive ability-screening instrument (CASI) (Teng et al., 1994) were used as a global assessment of cognitive function. Attention, verbal fluency, abstract thinking, and mental manipulation subdomain scores of the CASI were summarized and treated as executive function test scores (Huang et al., 2013). The nonexecutive subdomains of the CASI included orientation, short- and long-term memory, language ability, and drawing.

Genotyping for MTHFR C677T

Genomic DNA was extracted from blood samples using a commercial kit (Genta Systems, Minneapolis, MN, US). The primers and probes were designed using SpectroDESIGNER software (Sequenom, San Diego, US). A multiplex polymerase chain reaction was performed, in which unincorporated double-stranded nucleotide triphosphate bases were dephosphorylated with shrimp alkaline phosphatase (Hoffman-LaRoche, Basel) followed by primer extension. The purified primer extension reaction was spotted onto a 384-element silicon chip (SpectroCHIP, Sequenom, San Diego, US). The resulting spectra were processed using SpectroREADER mass spectrometer (Sequenom, San Diego, US). The apolipoprotein E (ApoE) genotype was also determined using a commercial kit (Gentra Systems, Minneapolis, MN, US). The primers and probes were designed using SpectroDesigner software (Sequenom, San Diego, US). A multiplex primer extension reaction was performed, in which unincorporated double-stranded nucleotide triphosphate bases were dephosphorylated with shrimp alkaline phosphatase (Hoffman-LaRoche, Basel) followed by primer extension. The purified primer extension reaction was spotted onto a 384-element silicon chip (SpectroCHIP, Sequenom, San Diego, US) and analyzed in a BrukerBiFlex III MALDI-TOF SpectroREADER mass spectrometer (Sequenom, San Diego, US). The resulting spectra were processed using SpectroTYPEPER (Sequenom, San Diego, US). The apolipoprotein E (ApoE) genotype was also determined using a PCR-restriction fragment length polymorphism assay and the restriction enzyme Hhal (Del Bo et al., 1997). ApoE4 carriers were defined as those with 1 or 2 E4 alleles (Huang et al., 2015).

Cerebrovascular Risk Confounders

Factors including oxidative stress, deregulated neuroinflammation, and metabolic derangements have been reported to be related to a greater WMH load in patients with AD (Huang et al., 2015). Therefore, the following risk confounders that may have a clinical impact were analyzed separately in each genotype group: hemoglobin, age, high sensitive C-reactive protein, homocysteine, total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, creatinine, vitamin B12, folate, and homocysteine (Hachinski ischemic score >4, and depression.

Image Acquisition

Magnetic resonance images were acquired using a 3.0 T MRI scanner (Excite, GE Medical Systems, Milwaukee, WI, USA). Structural images were acquired for SCN construction following the following protocol: a T1-weighted, inversion-recovery-prepared, three-dimensional, gradient-recalled acquisition in a steady-state sequence with a repetition time/echo time/inversion time of 8,600 ms/190 ms/450 ms, a 256 × 256 mm field of view, and a 1-mm slice sagittal thickness with a resolution of 0.5 × 0.5 × 1 mm³. The whole brain was acquired in 4 min 41 s.

Diffusion tensor images were acquired using the following parameters: repetition time/echo time/flip angle = 9,600 ms/90°, a 192 × 192 mm field of view, a 128 × 128 matrix and a 4-mm axial slice thickness. For whole brain coverage, 40 contiguous axial slices were obtained. Diffusion-weighted gradients were applied in 61 non-collinear directions and optimized using a static electron-repulsion model. The b value used was 1,000 s/mm². One reference image was acquired using the same imaging parameters but without diffusion weighting. The acquisition time was 10 minutes and 15 seconds.

Data Analysis for Neuroimaging Biomarkers

Image preprocessing and statistical analyses were performed using SPM8 software (SPM8, Wellcome Trust Centre of Cognitive Neurology, University College London, UK, http://www.fil.ion.ucl.ac.uk/spm/). The T1 images were reoriented, realigned, and normalized using the standard Montreal Neurological Institute space. The images were then segmented into GM and white matter (WM). Related tissue segments were used to create a custom template using diffeomorphic anatomical registration using an exponentiated lie algebra approach that represents one of the highest ranking registration methods in patients with AD (Cuingnet et al., 2011). The modulated and warped images were then smoothed using a Gaussian kernel of 8 mm full width at half maximum.

To investigate the SCNs, four regions of interest, representing seeds, were selected from the 135 preprocessed images. The following seeds that anchor the DMN medial temporal subsystem (right entorhinal cortex [coordinates: 25, -9, -28]) (Bernhardt, Worsley et al., 2008), DMN midline core subsystem (left posterior cingulate cortex [PCC; coordinates: -2, -36, 35]) (Spreng and Turner, 2013; Zielinski et al., 2012), salience network (right frontoinsular cortex [coordinates: 38, 26, -10]), and executive control network (right dorsolateral prefrontal cortex [coordinates: 44, 36, 20]) (Seeley et al., 2007) were selected (Fig. 1A). As the pathology or functional connectivity in typical patients with AD is distributed symmetrically, we did not perform contralateral seed analysis in this study.

From the modified GM images, the GM volumes of a 4-mm radius sphere around the seed coordinates were also calculated, followed by four separate correlation analyses.
analyses using the extracted GM volumes as the covariates of interest. The T homozygous variant allele was extremely rare in the study population (TT n = 6). Therefore, carriers of the heterozygous CT (n = 56) and homozygous TT variants were combined into a T carrier group for the analysis. There were 73 cases with C homozygotes.

The two genotype groups were modeled separately. In each group, specific contrasts were set to identify voxels that showed positive correlations for each seed. The results reflected the SCNs of each seed, and the threshold was set at $p < 0.01$, corrected for a false discovery rate with a cluster size $> 100$ voxels.

In addition, to investigate how genetic variance may interfere with SCN covariance strength, voxels showing significant differences in the regression slopes of each seed-peak cluster correlation that suggested interactions between CC $>$ T-carriers or CC $<$ T-carriers were compared. Specific $t$ contrasts were established to map the voxels that expressed significant between-group associations. The threshold for the resulting statistical parametric maps was set at $p < 0.001$ (uncorrected) with a cluster size $> 100$ voxels. The rationale for this cluster size was to avoid possible false-positive results. For the peak clusters showing significant between-group differences, a 4-mm radius sphere was placed on the peak voxel, and the GM volumes were then calculated for regression analysis. To evaluate the clinical significance of the seed or identified peak voxel, we used partial correlation analysis with the cognitive test scores adjusted for serum biomarkers levels. The threshold was set at $p < 0.05$ with multiple corrections.

**WM Tract Parcellation and Comparisons**

We also evaluated whether these changes in SCN pattern reflected the WM-integrity differences between two genotype groups. In brief, the tract probabilistic maps were used as templates for automated parcellation of the WM. The JHU-DTI template and averaged fiber probability maps were used (http://lbam.med.jhmi.edu). The WM parcellation algorithm and calculation of 11 major bundles followed the procedure reported by Hua et al. (Hua et al., 2008). The neuroimaging parameters used to assess the macro-structural integrities of the WM bundles were derived from the diffusion tensor images, namely the fractional anisotropy, mean diffusivity, axial diffusivity and radial diffusivity maps. Differences in WM integrity were compared between the two genotype groups.

**Statistical Analysis**

Clinical and laboratory data were expressed as mean ± standard deviation. The student’s $t$ test was used to compare levels of cerebrovascular risk biomarkers or continuous variables of the C-homozygotes and T-carriers. All statistical analyses were conducted using SPSS software (SPSS version 22 for Windows®, SPSS Inc., Chicago, IL). Statistical significance was set at $p < 0.05$.

**RESULTS**

**Demographic Data and Cognitive Data**

There were no significant differences in gender, age, educational level or ApoE4 status between the two genotype groups.

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**Figure 1.**

Statistical maps depicting brain areas in which the gray matter intensity covaried with (A) four target seeds, (B) seed volume comparisons, and (C) structural covariance networks (Z-statistic maps [$p < 0.01$, corrected with a false discovery rate with extended cluster voxels $> 100$]) in all the enrolled Alzheimer’s disease patients with the methylenetetrahydrofolate reductase CC ($n = 73$, red color) or T ($n = 62$, blue color) genotype. A significantly lower posterior cingulate cortex gray matter seed volume was found in the T carriers ($p < 0.05$). The images are displayed on a standard brain render. (D) Scatter plot between seed volumes and homocysteine levels. [Color figure can be viewed at wileyonlinelibrary.com]
groups, however significantly higher levels of homocysteine and creatinine, and a lower eGFR were found in the T-carriers (Table I). Among the patients, there were 30 patients showing stage III (GFR 30–59), 74 patients with CKD stage II (GFR 60–89) and 31 with stage I (GFR >90, 1.73m²). In correlation analysis, homocysteine level was significantly associated with eGFR (r = -0.476), and levels of high-density lipoprotein (r = -0.476), folate (r = -0.403) and vitamin B12 (r = -0.356) (all p < 0.001).

SCN Patterns

The SCN patterns are shown in Figure 1. Among the preselected seeds (Fig. 1A), a significantly smaller GM volume of the PCC seed was found in the T-carriers (Fig. 1B, p < 0.05). Networks showing structural associations with each seed region in the two genotype groups are shown in Figure 1C and Supporting Information, Tables I–VIII. The correlation between PCC seed volume and homocysteine level was significant (r = -0.378, p < 0.001), however this relationship was not found in the seed volumes of the entorhinal cortex (r = 0.016, p = 0.857), frontoinsular cortex (r = -0.136, p = 0.118) or dorsolateral cortex (r = -0.07, p = 0.423) (Fig. 1D).

TABLE I. Demographical characteristics and neuropsychiatric tests in the MTHFR genotype groups in 135 Alzheimer’s disease

| Group                   | C homozygotes (n = 73) | T carriers (n = 62) | P value |
|-------------------------|------------------------|---------------------|---------|
| Age                     | 73.47 (6.51)           | 73.23 (8.69)        | 0.86    |
| Education (year)        | 6.62 (4.65)            | 8.21 (4.90)         | 0.06    |
| Apolipoprotein E4 carrier (positive case, %) | 35, 47.9%          | 24, 38.7%           | 0.301   |
| Sex (male/female)       | 30/43                  | 35/27               | 0.086   |
| Mini-Mental State Examination | 20.55 (6.00)      | 20.02 (7.10)        | 0.64    |
| CASI total scores       | 69.19 (18.86)          | 66.31 (23.66)       | 0.43    |
| CASI executive function test scores | 25.89 (7.02)   | 24.56 (9.66)        | 0.36    |
| CASI subdomains         |                        |                     |         |
| Short-term memory       | 5.30 (3.77)            | 5.69 (3.76)         | 0.54    |
| Orientation             | 13.36 (5.03)           | 12.11 (5.47)        | 0.17    |
| Long-term memory        | 8.40 (2.52)            | 8.23 (2.46)         | 0.69    |
| Language                | 8.20 (2.08)            | 8.07 (2.47)         | 0.75    |
| Drawing                 | 8.19 (2.56)            | 7.65 (3.00)         | 0.26    |
| Attention               | 6.41 (1.09)            | 6.23 (1.74)         | 0.45    |
| Verbal fluency          | 5.29 (2.63)            | 5.15 (3.02)         | 0.77    |
| Abstract thinking       | 8.48 (2.46)            | 7.71 (3.28)         | 0.12    |
| Mental manipulation     | 5.71 (3.16)            | 5.48 (3.40)         | 0.69    |
| Cerebrovascular risk biomarkers |                |                     |         |
| High-sensitive C-reactive protein (mg/L) | 2.99 (5.50)       | 2.37 (4.16)         | 0.48    |
| Homocysteine (umol/L)   | 11.45 (3.62)           | 14.01 (4.81)        | 0.001   |
| Hemoglobin-A1C (%)      | 6.12 (0.96)            | 6.18 (1.34)         | 0.78    |
| Creatinine (mg%)        | 0.89 (0.21)            | 1.03 (0.52)         | 0.01    |
| Glomerular filtration rate (mL/min) | 78.84 (20.07)    | 70.57 (20.67)       | 0.02    |
| High-density lipoprotein (mg/dl) | 57.94 (15.34)   | 57.82 (18.77)       | 0.97    |
| Low-density lipoprotein (mg/dl) | 104.14 (39.60)  | 104.66 (32.87)      | 0.93    |
| Hemoglobin (mg/dl)      | 13.49 (1.45)           | 13.19 (1.71)        | 0.27    |
| Total cholesterol (mg/dl) | 187.88 (39.59)   | 185.82 (40.51)      | 0.77    |
| Triglyceride (mg/dl)    | 120.42 (62.11)        | 116.71 (59.16)      | 0.73    |
| Vitamin B12 (pg/dl)     | 638.44 (350.75)       | 573.49 (329.02)     | 0.28    |
| Folate (ng/dl)          | 12.96 (5.46)          | 11.27 (5.61)        | 0.08    |

Data are presented as mean (standard deviation) or number (percentage; %). Abbreviations: CASI, Cognitive Ability Screening Instrument; MTHFR, methylenetetrahydrofolate reductase. Attention, verbal fluency, abstract thinking, and mental manipulation subdomain scores of the CASI were added to assess executive function; APOE4 carriers were defined as the presence of one or two APOE4 alleles. The Glomerular filtration rate is calculated by the Modification of Diet in Renal Disease (MDRD) formula.
Covariance strength showing interactions between 2 MTHFR genotype groups

Figure 2.

Seed and peak clusters volume showing significant interactions in covariance strength between the two genotype groups. Peak clusters of $CC > T$-carriers connected to the seeds are shown in 2A or 2D (orange circles). Peak clusters of $CC < T$-carriers connected to the seeds are shown in 2B, 2C, or 2E (yellow circles). Bar graphs represent the correlation coefficients between each seed and peak cluster volumes. A direct comparison of 10 peak cluster volumes between the two genotype groups was not significant (2F). Abbreviations: entorhinal cortex (EC), posterior cingulate cortex (PCC), and frontoinsular (FI) seed. $(x,y,z) =$ Montreal Neurological Institute coordinates. [Color figure can be viewed at wileyonlinelibrary.com]

Serum Biomarkers That Predict Cognitive Outcomes in Each Genotype

We then entered all the predefined cerebrovascular risk biomarkers into the regression model and analyzed the significance of these variables for predicting each cognitive measure in each genotype group (Table III). In the C homozygotes, the only significant variable that may have confounded the cognitive test score was hemoglobin level. In contrast, the significant confounding variables in the T-carriers included levels of hemoglobin, high-sensitive C-reactive protein, and vitamin B12. Although the homocysteine levels were different between the two genotype groups (Table I), the homocysteine level per se was not correlated with any of the cognitive test scores.

Seed Region Volumes and Relationships with Cognitive Scores

In each genotype group and seed volume, partial correlation analysis between the seed region volume and cognitive test scores was performed (Table IV), adjusted for the confounders identified in Table III. Although the homocysteine level was not significantly correlated with any of the cognitive test scores, we also tested whether the homocysteine level varied with the seed volume to predict the cognitive test outcomes. The rationale for adjusting for homocysteine level was based on differences in homocysteine levels between the genotype groups. Compared with the C homozygote group, there were more significant correlations between the seed region volumes and cognitive...
test scores in the T-carriers. In addition, the correlation coefficients in the T-carriers increased after adjusting for homocysteine level, compared to a decrease in the C homozygotes. There were no significant correlations between the cognitive tests and entorhinal cortex seed or dorsolateral prefrontal seed volumes.

**Clinical Significance of Entorhinal Cortex or PCC Seed-Related Peak Clusters Showing Genotype Differences**

In covariance strength CC > T-carriers, there were no significant correlations in peak cluster volumes and MMSE,

**TABLE II. Connectivity interactions of MTHFR genotypes with predefined seeds**

| Seed                  | Peak regions                          | Side | x   | y   | z   | Extent | MaxT  | P value  |
|-----------------------|---------------------------------------|------|-----|-----|-----|--------|-------|----------|
| CC > T carrier        |                                       |      |     |     |     |        |       |          |
| Entorhinal seed       | Frontal_Mid                           | R    | 26  | 44  | 25  | 108    | 3.81  | <0.0001  |
| PCC seed              |                                       |      |     |     |     |        |       |          |
| Frontoinsular seed    | calcarine                             | R    | 15  | -67 | 6   | 119    | 3.57  | <0.0001  |
| Dorsolateral prefrontal|                                       |      |     |     |     |        |       |          |
| CC < T carrier        |                                       |      |     |     |     |        |       |          |
| Entorhinal seed       | Paracentral lobule                    | L    | -6  | -22 | 58  | 294    | 4.51  | <0.0001  |
| Midcingulum           | L                                     | -2   | -13 | 43  | 409   | 3.8   | <0.0001  |
| Precuneus             | L                                     | 2    | -66 | 43  | 141   | 3.74  | <0.0001  |
| Superior temporal pole| R                                     | 54   | 6   | -17 | 164   | 3.83  | <0.0001  |
| Precuneus             | R                                     | 14   | -57 | 21  | 100   | 3.72  | <0.0001  |
| Mid-temporal          | R                                     | 62   | -9  | -14 | 174   | 3.7   | <0.0001  |
| Frontoinsular seed    | Parahippocampus                       | R    | 20  | -28 | -14 | 106    | 3.96  | <0.0001  |
| Dorsolateral prefrontal|                                   |      |     |     |     |        |       |          |

Peak regions are within the main cluster; R = right, L = left.
Max T is the maximum T statistic for each local maximum. P < 0.001 with cluster size = 100.
NA, not available; PCC, posterior cingulate cortex; MTHFR, methylenetetrahydrofolate reductase.

**TABLE III. Significant laboratory data in each MTHFR genotype for predicting the cognitive outcomes**

| Cognitive measures                  | C homozygotes | T carriers |
|-------------------------------------|---------------|------------|
|                                     | Significant variable | Standardized β | P value | Significant variable | Standardized β | P value |
| Mini-Mental State Examination       | Hb            | 0.404      | 0.005    | Hb          | 0.451  | 0.003   |
| CASI total scores                   | Hb            | 0.351      | 0.017    | Hb          | 0.325  | 0.038   |
| CASI executive function test scores | Hb            | 0.383      | 0.009    | hsCRP       | 0.282  | 0.04    |
| CASI subdomains                     |               |            |          | hsCRP       | 0.277  | 0.043   |
| Short-term memory                   | NA            | -          | -        | hsCRP       | 0.283  | 0.046   |
| Orientation                         | NA            | -          | -        | B12         | 0.307  | 0.05    |
| Long-term memory                    | NA            | -          | -        | Hb          | 0.394  | 0.015   |
| Language                            | Hb            | 0.331      | 0.017    | Hb          | 0.362  | 0.020   |
| Drawing                             | NA            | -          | -        | NA          | -      | -       |
| Attention                           | Hb            | 0.387      | 0.007    | NA          | -      | -       |
| Verbal fluency                      | NA            | -          | -        | hsCRP       | 0.284  | 0.044   |
| Abstract thinking                  | NA            | -          | -        | Hb          | 0.295  | 0.048   |
| Mental manipulation                 | Hb            | 0.286      | 0.043    | NA          | -      | -       |

The analysis is performed by Linear Regression Model using independent variables (Predictors) including high-sensitive C-reactive protein (hsCRP), hemoglobin A1c (HbA1c), glomerular filtration rate (GFR), HDL, LDL, total cholesterol, hemoglobin (Hb), B12, folate, and homocysteine (Hcy); CASI, Cognitive Ability Screening Instrument; NA, not available.
CASI total scores, and short-term memory cognitive test scores (Table V). In covariance strength T-carriers > CC, more peak clusters showed significant correlations between the volume and cognitive test scores, and this was especially pronounced in the T-carriers. Interestingly, although the entorhinal cortex seed volume did not predict the cognitive outcomes, the interconnected peak clusters correlated with the MMSE or CASI total scores differed in the C homozygotes (paracentral lobule) and T-carriers (paracentral lobule, mid-cingulum, and precuneus).

Changes of GM SCN Network Independently of WM Integrity

A direct comparison between the two genotype groups in 11 major fiber tract macrointegrities using four diffusion

### Table IV. Partial correlation analysis between cognitive test scores and seed volumes

| MTHFR genotypes                  | C homozygotes | T carriers | C homozygotes | T carriers |
|----------------------------------|---------------|------------|---------------|------------|
|                                  | Left posterior cingulate cortex seed | Right frontoinsular cortex seed | Left posterior cingulate cortex seed | Right frontoinsular cortex seed |
| MMSE                             | Volume<sup>a</sup> | Volume<sup>b</sup> | Volume<sup>a</sup> | Volume<sup>b</sup> |
| MMSE                             | 0.276<sup>*</sup> | 0.24<sup>*</sup> | 0.327<sup>**</sup> | 0.37<sup>**</sup> |
| CASI total scores                | 0.231         | 0.22       | 0.309<sup>**</sup> | 0.38<sup>**</sup> |
| CASI EFT scores                 | 0.164         | 0.12       | 0.312<sup>**</sup> | 0.38<sup>**</sup> |
| CASI Subdomains                 |               |            |               |            |
| Short-term memory               | Volume<sup>a</sup> | Volume<sup>b</sup> | Volume<sup>a</sup> | Volume<sup>b</sup> |
| Orientation                      | 0.25<sup>*</sup> | 0.23<sup>*</sup> | 0.318<sup>**</sup> | 0.38<sup>**</sup> |
| Long-term memory                 | 0.12          | 0.12       | 0.262<sup>**</sup> | 0.33<sup>**</sup> |
| Language                         | 0.263<sup>**</sup> | 0.23       | 0.32<sup>**</sup> | 0.36<sup>**</sup> |
| Drawing                          | 0.04          | 0.07       | 0.15          | 0.19       |
| Attention                         | 0.193         | 0.08       | 0.39<sup>**</sup> | 0.42<sup>**</sup> |
| Verbal fluency                   | 0.20          | 0.18       | 0.253         | 0.27<sup>**</sup> |
| Abstract thinking                | 0.06          | -0.02      | 0.263<sup>**</sup> | 0.32<sup>**</sup> |
| Mental manipulation              | 0.118         | 0.09       | 0.27<sup>**</sup> | 0.30<sup>**</sup> |

Numbers indicate Pearson correlation coefficients, *<sup>P</sup> < 0.05; **<sup>P</sup> < 0.01. Volume<sup>a</sup>, adjusted for confounders based on Table III results; Volume<sup>b</sup>, adjusted for homocysteine level. MMSE, Mini-Mental State Examination; CASI, Cognitive Ability Screening Instrument; EFT, executive function test; MTHFR, methylenetetrahydrofolate reductase.

There are no significant correlations between the cognitive tests and entorhinal seed (or dorsolateral prefrontal lobe seed) volume.

### Table V. Partial correlation between Peak cluster volume and cognitive measures in MTHFR genotypes adjusted for laboratory confounders

| Structural covariance networks | C homozygotes | T carriers | C homozygotes | T carriers |
|--------------------------------|---------------|------------|---------------|------------|
|                                  | MMSE         | CASI       | CASI STM      | MMSE       | CASI       | CASI STM      |
| Seed                            |              |            |               |            |            |               |
| Entorhinal seed                  |              |            |               |            |            |               |
| CC > T carrier                   |              |            |               |            |            |               |
| Mid-Frontal                      | -0.01        | -0.067     | -0.042        | 0.192      | 0.236      | 0.091         |
| CC < T carrier                   | -0.031       | 0.044      | -0.097        | 0.074      | 0.064      | -0.033        |
| Entorhinal seed                  |              |            |               |            |            |               |
| Paracentral lobule               | 0.298<sup>*</sup> | 0.257<sup>**</sup> | 0.221        | 0.279<sup>*</sup> | 0.316<sup>*</sup> | 0.109        |
| Mid-cingulum                     | 0.203        | 0.182      | 0.198         | 0.255<sup>**</sup> | 0.220      | 0.080         |
| Precuneus                        | 0.061        | 0.066      | 0.068         | 0.32<sup>**</sup> | 0.309<sup>**</sup> | 0.198        |
| Inferior temporal                | -0.068       | -0.029     | 0.177         | 0.435<sup>**</sup> | 0.411<sup>**</sup> | 0.316<sup>**</sup> |
| Superior temporal polar          | 0.096        | 0.085      | 0.079         | 0.305<sup>**</sup> | 0.188      | 0.227         |
| Precuneus                        | 0.208        | 0.218      | 0.2           | 0.52<sup>**</sup> | 0.529<sup>**</sup> | 0.395<sup>**</sup> |
| Mid-temporal                     | 0.135        | 0.070      | 0.121         | 0.051      | 0.079      | -0.086        |
| Frontoinsular seed               | 0.079        | 0.103      | -0.074        | 0.276<sup>**</sup> | 0.203      | 0.124         |

Abbreviations: MMSE, Mini-Mental State Examination; CASI, Cognitive Ability Screening Instrument; MTHFR, methylenetetrahydrofolate reductase; STM, short-term memory; Adjusted parameters based on the significant confounders as shown in Table III; *<sup>P</sup> < 0.05; **<sup>P</sup> < 0.01.
DISCUSSION

Major Findings

The results of this study provide an insight into the network-specific genetic influence of the MTHFR C677T functional polymorphism in the early stages of AD. There were three main findings. First, the seed-based SCN pattern validated the hypothesis that this MTHFR functional polymorphism targets the PCC-anchored brain networks. In comparisons of covariance strength, more clusters showing significant clinical correlations in both PCC seed volume and PCC interconnected peak cluster volume were found in the T-carriers. Second, although the T-carriers had higher serum levels of homocysteine, the WM microstructural integrity was not different from the C homozygotes, suggesting that genetic modulation of the GM network may be independent from the impact of WMHs. Third, analysis of the serum confounding factors on cognitive outcomes implied the importance of hemoglobin level in both genotype groups. In the T-carriers, levels of high-sensitive C-reactive protein and B12 were significantly associated with cognitive outcomes, suggesting that cognitive performance is related to both genetic and biomarker modulation.

Genotype Interaction on PCC-Anchored SCN in T-Carriers

In the T-carriers, we found a smaller PCC GM volume, higher serum homocysteine level, and a correlation between PCC GM volume and homocysteine level. In cognitive test correlations, we also found that the PCC seed volume had a greater cognitive predictive value than the other three seeds. PCC remains an early target of amyloid deposition both in patients with AD (Li et al., 2008) and in cognitively normal subjects with a positive family history of AD (Mosconi et al., 2010). Based on these findings, the changes in PCC-anchored SCN in each MTHFR genotype may be mediated directly by genetic–homocysteine interference. Previous studies have also suggested that an elevated plasma homocysteine concentration is associated with an increase in Aβ1-42 deposition in the brain (Kamat et al., 2015; Li et al., 2014). Indirect evidence of the possible impact of hyperhomocysteinemia on the densely amyloid-packed PCC volume was also supported by our correlation analysis of PCC volume and homocysteine level in the T-carriers.

Stronger covariance strength between the seed and peak clusters has been reported to indicate more intranetwork connections (Lin et al., 2016). We found more clusters in the T-carriers showing stronger covariance than the C homozygotes, especially in the PCC interconnected regions. The PCC seed volume and PCC-anchored peak cluster volumes parameters showed no statistically significant differences (Table VI).

MTHFR, methylenetetrahydrofolate reductase; no difference between two genotype groups in any of the diffusion parameter values.

| Fiber name                      | Genotypes       | Fractional anisotropy | Axial diffusivity ($\times 10^{-3}$) | Radial diffusivity ($\times 10^{-3}$) | Mean diffusivity ($\times 10^{-3}$) |
|---------------------------------|-----------------|-----------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| Forceps major                   | C Homozygotes   | 0.48 ± 0.042          | 1.48 ± 0.13                         | 0.69 ± 0.13                         | 0.95 ± 0.13                         |
|                                 | T Carriers      | 0.47 ± 0.040          | 1.49 ± 0.14                         | 0.70 ± 0.12                         | 0.96 ± 0.12                         |
| Forceps minor                   | C Homozygotes   | 0.35 ± 0.025          | 1.41 ± 0.06                         | 0.81 ± 0.07                         | 1.01 ± 0.06                         |
|                                 | T Carriers      | 0.35 ± 0.027          | 1.42 ± 0.07                         | 0.82 ± 0.07                         | 1.02 ± 0.07                         |
| Anterior thalamic radiation     | C Homozygotes   | 0.61 ± 0.054          | 3.03 ± 0.42                         | 2.03 ± 0.39                         | 2.36 ± 0.40                         |
|                                 | T Carriers      | 0.60 ± 0.059          | 3.06 ± 0.47                         | 2.06 ± 0.44                         | 2.40 ± 0.45                         |
| Corticospinal tract             | C Homozygotes   | 1.00 ± 0.055          | 2.61 ± 0.11                         | 1.18 ± 0.12                         | 1.66 ± 0.11                         |
|                                 | T Carriers      | 0.99 ± 0.055          | 2.62 ± 0.11                         | 1.20 ± 0.12                         | 1.67 ± 0.11                         |
| Cingulum cingulate gyrus        | C Homozygotes   | 0.68 ± 0.059          | 2.28 ± 0.09                         | 1.37 ± 0.10                         | 1.67 ± 0.09                         |
|                                 | T Carriers      | 0.67 ± 0.064          | 2.27 ± 0.09                         | 1.38 ± 0.11                         | 1.68 ± 0.10                         |
| Cingulum hippocampus            | C Homozygotes   | 0.59 ± 0.075          | 2.31 ± 0.22                         | 1.50 ± 0.21                         | 1.77 ± 0.21                         |
|                                 | T Carriers      | 0.57 ± 0.064          | 2.38 ± 0.27                         | 1.57 ± 0.25                         | 1.84 ± 0.26                         |
| Inferior frontooccipital fasciculus | C Homozygotes | 0.74 ± 0.052          | 2.55 ± 0.13                         | 1.42 ± 0.14                         | 1.80 ± 0.13                         |
|                                 | T Carriers      | 0.74 ± 0.055          | 2.56 ± 0.14                         | 1.44 ± 0.16                         | 1.81 ± 0.15                         |
| Inferior longitudinal fasciculus | C Homozygotes  | 0.75 ± 0.051          | 2.41 ± 0.13                         | 1.34 ± 0.13                         | 1.70 ± 0.12                         |
|                                 | T Carriers      | 0.74 ± 0.047          | 2.42 ± 0.12                         | 1.35 ± 0.13                         | 1.71 ± 0.12                         |
| Superior longitudinal fasciculus | C Homozygotes  | 0.63 ± 0.051          | 2.28 ± 0.15                         | 1.45 ± 0.16                         | 1.73 ± 0.15                         |
|                                 | T Carriers      | 0.63 ± 0.045          | 2.27 ± 0.12                         | 1.45 ± 0.12                         | 1.73 ± 0.12                         |
| Uncinate fasciculus             | C Homozygotes   | 0.68 ± 0.048          | 2.65 ± 0.24                         | 1.61 ± 0.22                         | 1.96 ± 0.22                         |
|                                 | T Carriers      | 0.68 ± 0.052          | 2.67 ± 0.29                         | 1.63 ± 0.28                         | 1.98 ± 0.28                         |
| Superior longitudinal fasciculus temporal part | C Homozygotes | 0.91 ± 0.090          | 2.46 ± 0.17                         | 1.17 ± 0.16                         | 1.60 ± 0.15                         |
|                                 | T Carriers      | 0.89 ± 0.078          | 2.48 ± 0.12                         | 1.19 ± 0.12                         | 1.62 ± 0.11                         |

TABLE VI. Comparisons of diffusion parameters in two MTHFR genotypes using 11 association Fibers
also significantly predicted the cognitive measures (Table IV), suggesting a greater clinical weighting of the MTHFR T polymorphism in the PCC-anchored DMN network. A previous neuroimaging study in subjects with schizophrenia, obsessive–compulsive disorder, or autism spectrum disorder showed a significant linear effect of MTHFR 677T allele load on dorsal anterior cingulate cortex activation (Agam et al., 2014). As the PCC and anterior cingulate cortex both represent early pathological targets in AD and are also highly functionally anchored, the MTHFR C677T genetic effects within the same network system but in different disease spectrums may reflect the common nature of a genetic mediating effect.

Another seed volume that showed group differences in correlation analysis in this study was the frontoinsular cortex seed (Table IV and Fig. 2D,E). Although the volumes between the two groups were comparable, the seed volume was significantly correlated with nearly all of the cognitive test scores in the T-carriers.

**MTHFR Genotypes and Cognition in Cognitively Normal Subjects or Patients With AD With Confounding Factors**

Gussekloo et al. (1999) reported that the C677T polymorphism was not a genetic risk factor for cognitive impairment in the elderly, and several follow-up studies have confirmed these observations (Almeida et al., 2005; Bathum et al., 2007; de Lau et al., 2010; Visscher et al., 2003). Elkins et al. (2007) suggested that subjects with the TT genotype may have decreased processing speed and executive functions, while Durga et al. (2006) reported controversial findings in that the T homozygotes showed better sensorimotor speed. In elderly Chinese males without dementia, those with the CT genotype have been reported to achieve higher CASI scores than those with the other two homozygotes in the short-term memory and mental manipulation subdomains (Tsai et al., 2011). Taken together, the genetic effects on cognitive measurements in cognitive normal elderly subjects do not seem to be consistent.

A recent meta-analysis and case–control studies have supported an association between MTHFR 677T and susceptibility to AD (Peng et al., 2015; Rai, 2017; Zhang et al., 2010). In this study, we found no significant differences in cognitive performance between the genotype groups. The lack of a genetic–clinical relationship may be related to the small sample size, as the C677T genetic effect on global cognitive function in the elderly has been reported to be mild to modest (Rui et al., 2010). In addition, we analyzed the possible confounding roles of serum biomarkers in each MTHFR functional group. Several studies have shown that the effect of the MTHFR genotype in predicting AD cognitive test outcomes can be confounded by factors including serum homocysteine level, ApoE genotype status (Polito et al., 2016), serum ApoE level (Roussotte et al., 2016), disease stage, in vivo amyloid burden, and endothelial integrity (Ma et al., 2016).

**MTHFR Genotypes and WMHs**

Homocysteine has been shown to exert multiple effects that may contribute to cognitive decline in patients with AD (Del Bo et al., 1997; Teng et al., 1994). These include increasing tau phosphorylation, glutamate receptor dysfunction, neuronal apoptosis induction, endoplasmic reticulum stress, DNA methylation, mitochondrial dysfunction, vascular damage (Huang et al., 2015), and oxidative stress (Moustafa et al., 2014). A previous study reported no significant associations between the MTHFR risk allele (677T) and WMHs (Rajagopalan et al., 2012), which is consistent with our findings. The adverse effects of plasma total homocysteine on cortical perfusion are toward a more anterior location (Huang et al., 2013). Recent pathology studies have implied that pure AD without vascular compromise is relatively uncommon, while a combination of subcortical vascular disease superimposed on a degenerative network is more common (Bronge and Wahlund, 2007). Although we found higher homocysteine levels in the T carriers, there were no differences between the two genotype groups in any preselected WM microstructure in this study. The presence of the T allele may not be completely detrimental based on the findings of positive heterosis in the subjects with the MTHFR polymorphism. A higher formyl to methyltetrahydrofolate derivative ratio in subjects with the T allele has been reported (Bagley and Selhub 1998), which may prevent cytotoxicity and apoptosis (Elkins et al., 2007; Zielinski et al., 2012).

**Confounding Risk Biomarkers Not Related to MTHFR Genotype**

Although the PCC interconnected network could be modulated by the genetic influence or effect of hyperhomocysteinemia, we found no direct predictive value of homocysteine level on the cognitive outcomes in either genotype group. In contrast, hemoglobin level had the best predictive effect, which may not have been related to the MTHFR functional polymorphism (Table III). It is also possible that the interference of hemoglobin on cognitive test scores was totally independent of homocysteine cascade, as the homocysteine level was only significantly associated with eGFR, high-density lipoprotein, folate, and vitamin B12 in this study.

**Study Limitations**

An important limitation of this study is that we did not include a control group, and our AD patients were at an early clinical stage. As the MTHFR genetic effect or effect of homocysteine level on brain structure can be confounded by AD pathological load (Kamat et al., 2015; Li et al., 2014), this may limit the application of the study results to healthy subjects or to patients with AD at advanced stages. Nonetheless, the results may help to explain the genotype–cognitive test
relationship in healthy elderly subjects. As the MTHFR genetic polymorphism in patients with AD leads to different serum homocysteine levels that interfere with selective seed volumes, SCN patterns, and covariance strength alterations, the cognitive test outcomes in healthy elderly subjects may reflect the consequence of brain network alterations. In addition, the composition of our study cohort is limited as we excluded those with stroke. This may limit the analysis of the genotype on WMH load related to extreme homocysteine levels. Meanwhile, there were 104 patients that had at chronic kidney disease stage II or III. Although the calculated results may have driven by the advanced aging in our AD, presence of chronic kidney disease may show interactions with the MTHFR genotype analysis. The sample size did not allow for further analysis of interactions among other genetic variants. For example, the effect of MTHFR 677T has been reported to increase WMH burden only in carriers with the ApoE E4 or E2 allele (Szolnoki et al., 2004). This study might be underpowered in comparing the difference of parameters between the group of C homozygotes and T carrier whose P values are equal to or over 0.05. For example, if the true difference of REC seed volume between those of C homozygotes and T carrier is 0.01 and z is set as 0.05, the power is as low as 0.073 (Fig. 1). The estimated sample size necessary to reach the power of 0.8 is 2628 in each group (balance design) and 3518 for a power of 0.9. As our study conclusions are mainly driven by the findings with statistical significance. Therefore, the consideration of the power issue would not change our study conclusions. Finally, as clinical significance was established in four predefined networks, we did not test whether other networks participated in this genetic modulation. The use of independent component analysis (Beckmann et al., 2005) or resting-state functional MRI data may elucidate other potential networks and also validate the findings observed in this study.

CONCLUSION
The MTHFR C677T polymorphism modulates the strength of structural covariance independently of WMHs in the early stages of AD. The 677T carriers had higher homocysteine levels related to q smaller PCC volume, and the T allele conveyed increased covariance strength target ing the PCC-anchored DMN networks that may then mod ulate the degenerative process differently from the patterns in C homozygotes.

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DISCLOSURE/CONFLICT OF INTEREST
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