Genetic variation in FOXP2 alters grey matter concentrations in schizophrenia patients

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A B S T R A C T

FOXP2, the first gene known to be involved in the development of speech and language, can be considered to be, a priori, a candidate gene in schizophrenia, given the mounting evidence that the underlying core deficit in this disease could be a failure of structures relevant to normal language processing. To investigate the potential link between grey matter concentration (GMC) changes in patients with schizophrenia and the FOXP2 rs2396753 polymorphism previously reported to be associated with hallucinations in schizophrenia, we analysed high-resolution anatomical magnetic resonance images of 40 genotyped patients with schizophrenia and 36 healthy controls, using optimised voxel-based morphometry (VBM). Here we show that the common SNP rs2396753 (C > A) gene variant of the FOXP2 gene has significant effects on GMC in patients with schizophrenia, within regions of the brain known to be affected by this disease. Our data suggest that GMC reductions in schizophrenia may be driven by C allele carriers of the FOXP2 gene variant.

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Despite intensive study, the molecular etiology of schizophrenia remains unknown. Altogether, the illness is most often conceptualized as a complex disease resulting from the cumulative impact of multiple small-effect, widely distributed genetic variants interacting with environmental exposure to exceed a biological threshold [20].

Promising heuristic methods applied to the genetics of complex disorders link known susceptible genetic variations to the phenotype of a given illness. In this respect, the emerging field of imaging genetics, which combines genetic and neuroimaging data, promises to provide fundamental changes to our understanding of the etiology of common diseases.

A growing body of evidence highlights a prominent role for MRI brain morphometry in the imaging genetics of schizophrenia, especially because brain morphometric alterations may represent an endophenotypic candidate for this disease [9,16]. Thus, the combination of structural MRI data with genetic research may be particularly useful for obtaining insight into the pathophysiology of schizophrenia.

Recent studies have suggested a relationship between brain structural alterations and variations in schizophrenia susceptibility genes, identified by genetic association studies (RGS4, NRG1, DISC1, PCM1), or candidate genes whose involvement has been hypothesised a priori (COMT, BDNF, Neurotrophin3, Interleukin-1, NOTCH4) [23].

The latter category of a priori schizophrenia candidate genes includes the FOXP2 gene as well [17]. Although an association between schizophrenia and FOXP2 was not reported in genetic association and linkage studies, its characteristics make the gene a compelling target for closer analysis.

FOXP2 (forkhead box P2) is located on chromosome 7q31, and its major splice form encodes a 715 amino acid protein belonging to the forkhead class of transcription factors [10]. In vivo verification of neural targets regulated by FOXP2 revealed a range of genes involved in diverse biological functions, including synaptic transmission, cell signalling, ion transport, neural development and modulation of plasticity in relevant neural circuits [3,24].

Mutations in FOXP2 are associated with a monogenic speech and language disorder [10,13,26]. This discovery of a link between FOXP2 and spoken language sparked investigations into possible
associations between the gene and vulnerability to mental disorders typified by language impairments, including autism and schizophrenia [6,15,17,22]. In fact, language deficits, such as poor verbal functioning and abnormal verbal production and comprehension, are common phenomenological traits in schizophrenia patients. Accordingly, over the past decade, in vivo neuroimaging techniques have led to the identification of structural aberrations in language-related pathways, as well as altered functional hemispheric lateralisation during speech production in schizophrenia patients. Moreover, studies of people at high risk for schizophrenia suggest that these findings may be under genetic control. Thus, deviations in the structure and function of language areas of the brain are likely to play important roles in the neurobiology of schizophrenia [11].

Because the verbal hallucinations which are a hallmark of psychosis may in fact represent a form of speech disorder [19], the association between FOXP2 polymorphisms and auditory hallucinations in schizophrenia has been previously studied [17]. A set of 14 single nucleotide polymorphisms, mainly located in the 5′ regulatory region of the FOXP2 gene, were investigated in a study population consisting of 186 schizophrenic patients with auditory hallucinations and 180 healthy controls. A statistically significant difference in the frequency of alleles with an excess of the C allele between schizophrenic patients with auditory hallucinations and healthy controls, was found in a single nucleotide polymorphism, rs2396753 (C>A), located in intron 2b of the FOXP2 gene (p = 0.027 after Bonferroni correction).

FOXP2 has been proposed to be involved in grey matter development within language related regions of the brain [11]. The evidence for structural deficits within similar regions of the brain in schizophrenia [11] justifies further investigation into the role(s) of FOXP2 in the neurobiology of this disease.

In this exploratory investigation, we asked whether FOXP2 rs2396753 affects the brain morphology of patients with schizophrenia. SNP rs2396753 was selected because it has been previously shown to be associated with auditory hallucinations; a prominent symptom of this illness.

In an attempt to answer this question, we performed high-resolution anatomical magnetic resonance imaging on genotyped schizophrenia (SZ) patients and healthy controls (HC), using optimised voxel-based morphometry (VBM).

Study subjects were recruited from the genetic and neuroimaging registry of the Prague Psychiatric Centre. Our study sample consisted of 40 patients and 36 healthy controls. For DSM-IV diagnosis, we used the Structured Clinical Interview SCID-Patient Version (SCID-I/P). All participants were right-handed (Edinburgh handedness inventory >60). Study subjects were Caucasians of Czech origin from 18 to 60 years of age. All study participants gave informed consent, and the local ethics committee approved this study.

Within 4 days after enrolment, all study participants were evaluated by MRI, as well as genotyping and psychometric measurements. Clinical symptoms were assessed using the positive and negative syndrome scale (PANSS). Experienced psychiatrists who performed these assessments were blind to the results of the genotyping and MRI investigations.

MRI images were obtained using a 1.5-T Magnetom Vision system (Siemens, Erlangen, Germany). Subjects were scanned using a T1-weighted 3D-SPGR sequence (repetition time TR of 2000 ms; echo time TE of 4.4 ms; bandwidth 130 Hz/pixel; field of view of 256 mm; matrix 256 × 256; slice thickness 1 mm; 160 contiguous sagittal slices; voxel size of 1 mm × 1 mm × 1 mm. and total acquisition time 8 min). Structural MRI analyses were performed using an optimised VBM protocol in SPM5 (http://www.filion.ucl.ac.uk/spm/software/spm5) implemented in MATLAB 7.3 (Math Works, Natick MA, USA). Briefly, each 3D volume of T1W images was segmented into grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF). Spatial normalisation parameters were estimated by matching GM tissue with the standard GM template provided by SPM5. These normalisation parameters were applied to the original T1W images. Optimally normalised T1W images were then segmented into GM, WM, and CSF segments. In order to restore tissue volumes modified during normalisation processing, Jacobian modulation was applied to normalised GM and WM segments. Modulated normalised GM, WM and CSF images were smoothed using a 10 mm full-width-at-half maximum (FWHM) Gaussian kernel.

Genomic DNA was isolated from blood samples using a JetQuick isolation kit (Genomed GmbH, Loehne, Germany). Extracted DNA samples were subjected to PCR to amplify a 140 bp target sequence containing the FOXP2 SNP (rs2396753). PCR primer sequences were TCAGGACTCAGGTAAGTGAAA (left) and [GC-clamp]-TGGCATGGATGATCCCTCG (right). The sequence of the fluoroscein [FL]-labelled GC-clamp was 6-FL- GGCGGGGCGGGCGGGGACGGGCGCGGGCGCGGGGACGGGCGCGGG. Allele typing was performed using cycling gradient capillary electrophoresis (CGE), a heteroduplex separation technique based on denaturing capillary electrophoresis [2,14]. All experiments were performed on a MegaBACE 1000 capillary-array genetic analyzer (GE Biosciences, Piscataway, NJ) equipped with a Caddy 1000 plate loading robot (Watrex Prague, Czech Republic) for automated overnight operation. Separations took place in a standard denaturing gel matrix (MegaBACE long-range matrix, Amersham Biosciences) containing 7 M urea. Running conditions included injection for 120 s at 3 kV and running at 6 kV for 90 min. The separation temperature was cycled between 48 °C and 50 °C at 1 min per cycle. Allele calling against an internal heterozygote reference standard was conducted using GeneMarker software (SoftGenetics, State College, PA).

Normalised and smoothed grey matter images were analysed using statistical parametric mapping within the framework of the General Linear Model in SPM5 software (Wellcome Department of Imaging Neuroscience, London, UK, 1994–2007).

VBM group differences were evaluated with group and SNP rs2396753 FOXP2 genotype as factors. Use of a full-factorial model allowed us to assess main group effects and the interaction between diagnostic group and genotype. Age and total brain volume (TBV) were included in the statistical analysis as covariates. In addition to the default F contrast, t contrasts were used in the evaluation of between-group differences in GMC as well.

To further elucidate the source of the effect resulting from the interaction between diagnostic groups and genotype, post-hoc t-tests (all combinations) were carried out in the second step, combining all inter- and intra-group contrasts between both healthy and schizophrenic AA, AC and CC genotype carriers. In total, 20 pair-wise exploratory t-test combinations were performed. These analyses included all combinations between AA, AC, and CC carriers in HC and between AA and AC carriers in the SZ group. A comparison with CC homozygote from the schizophrenia group was also performed (Fig. 1a). However, this was excluded from the main analysis, because the CC variant was represented by only 1 subject in the SZ group.

For all analyses in this study, a false discovery rate (FDR) correction for multiple comparisons was used with a p-value of <0.05 and a cluster (k) > 30 contiguous voxels. The Mann–Whitney U test, Fisher’s exact and Chi-square test were used to compare demographic data and clinical characteristics between schizophrenics and healthy controls, as well as different genotypes within the patient group. Results are presented as an exact p value. Hardy-Weinberg equilibrium (HWE) was calculated using a HWE calculator (http://www.genes.org.uk/software/hardy-weinberg.shtml).
Fig. 1. (a) A post hoc analysis examining all t-test contrasts between AA, CC homozygotes and AC heterozygotes in FOXP2 SNP rs2396753 (t-contrasts are shown as columns × rows). Single CC SZ subject comparisons are marked with an asterisk. (b) Rendered T-map of regions of the brain where schizophrenia patients had reduced GMC compared to healthy controls (c) GMC loss in AC allele carriers from the schizophrenia group compared to healthy controls. (d) Full factorial model: interaction between group and genotype FOXP2 SNP rs2396753. Results are displayed using a study-specific template and slices are derived from mean images of all normalised T1W images. Significance threshold with a false discovery rate (FDR) at p < 0.05, k > 30. HC = healthy control group, SZ = schizophrenia group.

Table 1 presents the sociodemographic and clinical characteristics of all study subjects. There were no differences in sex, handedness or education between the schizophrenic patients and healthy controls. However, there was a significant difference in age between both groups (p < 0.001). To address this incongruency, age was also included as a covariate within the full factorial model in addition to the standard total brain volume. In the schizophrenia group, AC and AA genotype groups did not differ in demographic and clinical variables (sex, age, diagnosis, duration of schizophrenia, handeness, present neuroleptic dosage, or PANSS, including P3 hallucinatory subscores). SNP rs2396753 was found to be in Hardy-Weinberg equilibrium (HWE) in healthy controls. In contrast, a deviation from HWE was found in the schizophrenia group (χ² = 9.74, p = 0.002), with a relative excess of AC heterozygotes.

We found reduced GMC in the SZ group compared to the HC group, mainly within the bilateral prefrontal and temporal cortices, with local maxima in the left dorsolateral prefrontal cortex (DLPFC) and extending to the amygdala, bilateral insula, anterior cingulate, and caudate.

Table 1
Sociodemographic and clinical characteristics of all study subjects.

<table>
<thead>
<tr>
<th>Patients (N=40)</th>
<th>Controls (N=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>8/3</td>
</tr>
<tr>
<td>Age, years. Mean (SD)</td>
<td>32.7 (10.8)</td>
</tr>
<tr>
<td>Education, years. Mean (SD)</td>
<td>11.8 (3.0)</td>
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<tr>
<td>Edinburgh handedness inventory. Mean (SD)</td>
<td>90.0 (10.9)</td>
</tr>
<tr>
<td>Duration of illness, years. Mean (SD)</td>
<td>3.4 (3.6)</td>
</tr>
<tr>
<td>Schizophrenia (N/%)</td>
<td>8/72.7</td>
</tr>
<tr>
<td>Schizophreniform disorder (N/%)</td>
<td>3/27.3</td>
</tr>
<tr>
<td>Schizoaffective disorder (N/%)</td>
<td>0</td>
</tr>
<tr>
<td>Present neuroleptic dosage. Chlorpromazine equivalents in mg/day. Mean (SD)</td>
<td>384.25 (257.8)</td>
</tr>
<tr>
<td>PANSS – P3 (hallucinatory). Mean (SD)</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>PANSS – N. Mean (SD)</td>
<td>9.4 (8.9)</td>
</tr>
<tr>
<td>PANSS – G. Mean (SD)</td>
<td>12.3 (10.4)</td>
</tr>
<tr>
<td>PANSS – T. Mean (SD)</td>
<td>23.6 (19.8)</td>
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<tr>
<td>FOXP2 SNP rs2396753 genotypes N (AA/AC/CC)</td>
<td>11/28/1</td>
</tr>
</tbody>
</table>

SD: standard deviation; NS: non-significant.

⁠† pCase control differences between schizophrenic SNP rs2396753 AA and AC carriers.

⁠‡ pDifferences between patients with schizophrenia and healthy controls. 

* p < 0.05.
Additional clusters were detected in the cerebellum, substantia nigra, bilateral parietal lobe and left occipital lobe (FDR corrected, \( p < 0.05 \)). No region was found to be significantly higher in GMC in schizophrenia patients compared to HC when corrected for multiple comparisons (Fig. 1b, Table 2-Supplementary data).

A full-factorial model allowed us to assess the interaction between diagnostic group and genotype. This analysis was performed by comparing the effect of genotype in healthy controls with the same effect in the schizophrenia group. This approach revealed a significant group x FOXP2 interaction in regions of the brain anatomically overlapping with areas displaying GMC reduction in the previous comparison: left DLPFC, bilateral parietal lobe, bilateral cerebellum, right basal ganglia, left prefrontal cortex, cingulate gyrus and right temporal lobe (FDR corrected, \( p < 0.01 \)). Additionally, a significant interaction was also identified in the left primary motor cortex, left-sided parahippocampal gyrus and right temporal lobe (FDR corrected, \( p < 0.05 \), Fig. 1c, Table 2-Supplementary data).

To further elucidate the source of the effect resulting from an interaction between diagnostic group and genotype, post-hoc \( t \)-tests (all combinations) were carried out in the second step combining all inter- and intra-group contrasts between AA, AC, CC genotype carriers (Fig. 1a). Note that the 1 schizophrenic CC homozygote was excluded from this analysis. However, for illustrative purposes only, Fig. 1a shows results for this subject as well.

Post-hoc analysis revealed profound, statistically significant reductions in GMC between AC heterozygotes from the SZ group and three other groups, namely: (i) SZ AC < HC AA, (ii) SZ AC < HC AC and even (iii) SZ AC < SZ AA. The other contrasts did not reveal any GMC differences using this approach.

A direct comparison between the HC group and separate AC heterozygotes from the SZ group showed GMC reductions in patients within regions overlapping those identified in an overall HC vs. SZ group analysis (Fig. 1c, Table 2-Supplementary data). Despite the reduced number of subjects in the SZ group, GMC deficits were far more extensive in separate SZ AC carriers, namely bilaterally in the perisylvian area, encompassing the vast majority of the prefrontal cortex and extending to anterior, median cingulate and paracingulate gyri on the medial surface. Profound GMC loss included much of bilateral temporal cortex (both the superolateral and medial temporal cortex) and the insula as well (FDR corrected, \( p < 0.01 \)). Additional clusters were detected in the cerebellum, substantia nigra, occipital and parietal lobe (FDR corrected, \( p < 0.05 \)). No significant GMC differences were observed in AA carriers from the schizophrenia group compared with healthy controls.

Our results strongly suggest a direct link between genetic variation in FOXP2 and brain morphometry deviations in patients with schizophrenia. GMC reductions in schizophrenia group were identified in FOXP2 SNP rs2396753 AC carriers in our sample.

In this respect, the deviation from HWE found in the schizophrenic group in favour of an increase in AC heterozygote frequency is particularly striking. This finding is unlikely to be due to genotyping bias, because no HWE deviation was observed in the control group. Although this deviation from HWE could be due to a sampling effect, given the possible association between rs2396753 AC heterozygosity and the illness itself, a selection effect cannot be completely ruled out.

Because CC homozygotes in the schizophrenia group were nearly absent (only 1 subject), a possible additive effect of C allele homozygosity on GMC in schizophrenia could not be evaluated. However, the only CC allele carrier with schizophrenia showed profound overall GMC reductions compared to all other groups. Furthermore, this finding was not observed in 5 CC healthy controls (Fig. 1a). This evidence, although inconclusive, points to the need for further study of grey matter alterations as a function of rs2396753C allele dosage in schizophrenia.

The possible mechanism by which FOXP gene variants may be associated with reduced GMC in schizophrenia patients remains unclear. However, as a transcription factor, FOXP2 regulates a large number of genes involved in neural development and function. These gene targets include FGF8, which plays a key role in cortical patterning, as well as HOXB5 and HOXB7, members of the homeobox family, and NEUROD2, SPOCK1 and PAX3c, which are involved in CNS development [18].

The biological role of the non-coding SNP rs2396753 remains unknown. It is unclear whether this phenotype-associated intronic SNP bears some functional role, or whether other variants within a linkage disequilibrium block are in fact true sources of the signal. However, there is mounting evidence suggesting that intronic regions are prominent reservoirs of regulatory non-protein-coding RNAs (ncRNAs), which may be involved in controlling gene expression [12].

The FOXP2 gene is critically involved in the development of speech and language capabilities in humans. In this respect, it is of particular note that C allele carriers from the schizophrenia group are prone to changes in the anatomy of the perisylvian area, the region of the brain subserving language functions. Those findings are consistent with MRI VBM meta-analyses of schizophrenia patients, which demonstrated that the most pronounced grey matter anomalies occur within brain areas bordering the sylvian fissure [4,5,7]. More specifically, primary language cortices were directly associated with a susceptible allele in our study. In a comparison between C allele carrying schizophrenics and healthy controls, the clusters with the most profound illness-related GMC reductions encompassed both Broca’s and Wernicke’s language areas and their contralateral homologues. Likewise, the interaction between the group and FOXP2 genotype variant showed peak GMC reductions within the pars triangularis of the left inferior frontal gyrus in close proximity to Broca’s area and, to a lesser extent, its contralateral counterpart as well.

The structure of the perisylvian area is under strong genetic control [21]. Despite being dispersed across different anatomical regions, the combined perisylvian cortex exhibits a common developmental genetic signature, in which FOXP2 expression is increased during CNS development [8]. This suggests that the perisylvian cortex may be developmentally hardwired for common involvement in specific human-cognitive functions, including language, and that FOXP2 and its regulatory network may play important roles in development and cortical patterning in this region of the brain. This hypothesis is further supported by the fact that a point mutation in FOXP2 is associated with several bilateral grey matter abnormalities in language-related and motor regions [1,25].

There are a number of limitations to the present study, including the small sample size, the use of a cross-sectional study design, and the application of multiple statistical comparisons. In addition, in this study we only present results from one SNP within the FOXP2 gene. Therefore, comprehensive sequencing of the relevant region in the FOXP2 gene and further genetic imaging studies to examine the synergic effects of other polymorphisms or haplotypes are warranted, to clarify whether SNP rs2396753 alone contributes to the pathophysiology of schizophrenia.

Our findings suggest that the intronic SNP rs2396753, or an adjacent locus in linkage disequilibrium, may influence the expression of the FOXP2 transcription factor, and hence affect the expression of downstream targets relevant to the development of perisylvian area in humans. Our results provide testable hypothesis for the influence of the C allele in SNP rs2396753 on the structure and function of language-related networks in healthy subjects. Likewise, those findings could serve as a basis for future empirical investiga-
tions aiming at confirming the vulnerability-conferring properties of the susceptible allele in schizophrenia and the identification of other putative risk factors leading, through an additive effect, to disease pathogenesis.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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Contributions: Dr. Spaniel takes responsibility for the integrity of the data and the accuracy of the data analysis and certifies that all authors had full access to all of the data included in this study. JH and FS designed the experiment. JT and II gathered MRI data. FS, JH, JC and MK collected data. FS, JH, TN performed general data processing and statistical analyses. FS wrote the paper in collaboration with JH and CH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.02.024.

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