Epigenetic and association study of *FOXP2* gene in schizophrenia

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Abstract

Background
Schizophrenia is considered a disease that only affects humans. Taking this into account, previous works have reported signals of positive selection specific to the human lineage for schizophrenia-associated genes. In the FOXP2 gene, there are two important features that contribute to consider this gene a likely candidate gene for schizophrenia vulnerability: FOXP2 is the first gene related to a language disorder, and it has been subject to positive selection in the human lineage.

Methods
We analyzed 27 SNPs located in the region of FOXP2 in a cohort of 293 patients with schizophrenia and 340 controls. In a subsample, the potential expansion of three trinucleotide tracts in FOXP2 was also screened. Methylation analysis of a CpG island located in the first exon of the gene was performed in post-mortem brain samples, as well as qRT-PCR analysis.

Results
No significant association between patients and controls was found. Nevertheless a significant association was found for SNP rs2253478 and the Poverty of speech (p=0.038 after Bonferroni correction). In patients a higher degree of methylation in the left parahippocampus gyrus was obtained.

Conclusions
No association of FOXP2 and schizophrenia was found. However a possible role of this gene in the language disorder in these patients cannot be ruled out. Epigenetic factors might be also involved in the developing of this disorder.
Background
It is widely accepted that neutral drift and Darwinian positive selection have played an important role in the evolution of human features. During the last few years, research has been focused on genome-wide scans of adaptatively evolving loci in the human genome to search for specific modern characteristics [1]. Although most of these characteristics are related to fitness, it has also been reported that some genes that are under positive selection in the human lineage can confer vulnerability to some diseases [2-4].

Schizophrenia is considered a disease related to the origin of Homo sapiens and could be a by-product of an adaptative process [3,5,6]. Previous reports have indicated a relationship between positively selected genes and schizophrenia. Crespi et al. found signals of positive selection in 28 of 76 schizophrenia candidate genes that had been previously reported as positive results in association studies [3]. Evidence of recent positive selection in the human lineage has also been found in haplotypes of MAOB and GABRB2 genes, which also confer an increased risk to schizophrenia [2,4]. Brain areas that are differentially dysregulated in schizophrenia include the regions that are most-notably subject to differential evolutionary change along the human lineage [7-9]. In addition, it has been recently suggested that metabolic processes altered in schizophrenia evolved at a higher rate in the human lineage when compared with the chimpanzee [10].

A selective advantage could affect the achievement of specific human capacities, such language. TJ Crow, postulates that schizophrenia is the price that Homo sapiens had to pay for the acquisition of language [9,11]. Moreover, recent neuroimaging studies report impairment in brain function relevant to language processing in individuals with schizophrenia and in those who are at a genetic risk for its development [12].
First evidence for a gene involved in language was described in 2001, when the *FOXP2* gene was identified by Lai et al. [13]. Identification of the transcriptional targets of FOXP2 revealed that this protein could regulate genes related to the development and function of the brain, genes under positive selection in human lineage and genes associated to schizophrenia [14]. Apart from the polyglutamine tracts, the human protein only differs in three amino acids from its ortholog in mouse, and two of these changes occurred in the human lineage after separation from the common ancestor shared with chimpanzees. Both changes are fixed in human populations, and there is evidence to support the fact that they have been under positive selection [15,16].

The study of polymorphisms in the *FOXP2* gene in association with the susceptibility to different pathologies of language impairment, such as specific language impairment, dyslexia or autism have not produced robust results [17], but the identification of two coding mutations related to verbal dyspraxia [18]. In schizophrenia, preliminary association studies have delivered controversial results [19-21]. To the best of our knowledge no methylation study of *FOXP2* has previously been done.

We hypothesized that *FOXP2* could be considered a candidate gene that may confer vulnerability to schizophrenia or to the language related symptoms of this disorder. To test this hypothesis, two different analyses were carried out: 1) an association study between *FOXP2* polymorphisms and schizophrenia and 2) the study of the methylation status of the *FOXP2* promoter in different areas of the brain in patients and controls.
Methods

Association study participants
For the association study, 293 patients and 340 healthy unrelated controls were analyzed. All patients and controls were Caucasians of Spanish descent. Exclusion criteria included organic brain syndromes, mental retardation, severe drug abuse, or inability to understand simple questions. Participants with previous psychiatric treatment were excluded as controls.

There were no significant differences in sex or age for both groups. All patients met DSM-IV criteria for schizophrenia. The Manchester scale [22], and the psychotic symptom rating scale (PSYRATS) [23], were used, respectively, to assess the clinical psychotic symptoms and the intensity of auditory hallucinations in all patients. The mean Manchester score was 8.79 (SD=5.56) and mean PSYRATS score was 16.26 (SD=23.26). This study was approved by the local Ethics Committee. All patients signed the informed consent form.

Post-mortem human brain samples
For methylation and expression analyses, human brain samples were kindly donated by the London Neurodegenerative Diseases Brain Bank at the Institute of Psychiatry. Grey tissue from both hemispheres of the superior temporal gyrus, parahippocampus gyrus and cingulate gyrus was obtained. For methylation analyses, one sample for each region was analyzed for both patient and control. For expression analyses, 13 samples from patients (6 from the right hemisphere and 7 from the left hemisphere) and 12 from controls (9 from the right and 3 from the left hemisphere) were analyzed.

Association study
Genomic DNA was extracted from peripheral blood leukocytes by the Puregene kit (Gentra Systems, MN, USA).
A total of 27 polymorphisms were analyzed, 10 of them by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and 17 by an iPLEX genotyping assay (Sequenom, CA, USA). Details of the primer sequences, PCR conditions and restriction enzymes are described in Supplementary Tables S1 and S2. Three regions were analyzed for potential trinucleotide expansions: two polyQ tracts of 40 and 10 residues, located in exons 5 and 6 of the gene, respectively, and a CGG-rich region in intron s1 close to the transcription start site. Primers flanking the three regions were designed (Supplementary Table S1). One primer in each pair was 5’-labeled with 6-FAM or HEX fluorophores. Fluorescent amplicons were electrophoresed with internal lane size standards in an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems Inc.) and fragment length polymorphisms analyzed with the GeneScan-v3.7 (Applied Biosystems, Inc.).

Statistical and genetic analyses were performed using Haploview v4.1, UNPHASED 3.10, and SSPS v13 software. Bonferroni correction was used for multiple tests. For the haplotype association study, four marker sliding-windows were used, with the exception of a five marker haplotype, for which association had been detected in a previous study [21].

**Methylation analysis**
DNA from brain samples was extracted using a Nucleon® Genomic DNA Extraction Kit (Tepnel Life Sciences). DNA from leukocyte samples was extracted using the Puregene kit (Gentra Systems).

DNA was fragmented with EcoRI (New England Biolabs) prior to overnight digestion with Proteinase K (Sigma Aldrich). DNA was cleaned, purified and concentrated using a Qiaex II kit (Qiagen). The processed DNA samples were treated with either
the CpGenome™ DNA Modification Kit (Chemicon® International) or the EpiTect Bisulfite Kit (Qiagen) in accordance with the supplier’s guidelines. DNA was amplified with specific primers for bisulphite-converted DNA (Supplementary Table S1). PCR fragments were cloned into the PCR 2.1 vector using the TOPO cloning kit (Invitrogen), or pGEM-T® vector using the pGEM-T® Easy Vector System (Promega), and sequenced with T7 and SP6 universal primers.

**Expression analysis**
Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen). Reverse transcription of 1 µg of RNA was performed using SuperScript™ III Reverse Transcriptase (Invitrogen) and random primer hexanucleotides (Promega). Quantitative RT-PCR was performed in triplicate for each sample on an iCycler iQ Real Time PCR System (Qiagen) with Power SYBR® Green PCR Master Mix (Applied Biosystems) using a standard protocol. Specific cDNA primers for FOXP2 and RPII, used as a control gene, were designed. Sequences and PCR conditions are shown in Table S1. The comparative CT method ($\Delta\Delta$CT) was used to measure the relative gene expression.

**Results**

**Association study**
Twenty-four intronic SNPs and three SNPs within the 5’ untranslated region of FOXP2 were selected for this study. SNPS rs13308496 and rs10254225 were monomorphic in our sample. In Figure 1, we show the location of the selected SNPs that were polymorphic in our samples. All of the SNPs were at Hardy-Weinberg equilibrium in both cases and controls, except for rs717233, which deviated from HWE in controls (Supplemental Table S3) and was removed from the selection in subsequent analyses. Allelic and genotypic frequencies of the twenty-four SNPs in
both cases and controls are shown in Table 1. Minor allele frequencies for the SNPs ranged between 0.02 and 0.49.

First, we conducted the single SNP association analysis. When all patients were included, we observed a significant association for SNP rs10447760 at allelic frequencies, although association did not remain after the conservative Bonferroni correction was applied. When comparing patients with auditory hallucinations versus controls (Supplementary Table S4), significant associations were found for SNP rs2396753 and SNP rs17137124. However, after Bonferroni correction was applied, the significant associations were lost.

When patients with auditory hallucinations were compared with patients without them (Supplementary Table S5), a significant association was found for SNP rs2253478 and SNP rs1456031 in genotype frequencies and for SNP rs2396753 in both, genotypic and allelic frequencies. However, once again, after Bonferroni correction was applied, the significant associations were lost.

Next, we carried out the haplotypic association analysis. Tests of the four marker haplotypes did not provide evidence of significant associations with schizophrenia or auditory hallucinations. However, when a five marker haplotype, which was found to be significant in a previous study [21], was analyzed, associations were detected for the same combination of alleles: rs7803667T/ rs10447760C/ rs923875A/ rs2396722C/ rs2396753A ($\chi^2=6.479; \ p=0.0109$). Interestingly, this combination of alleles was found more frequently in controls.

Linear regression was performed to evaluate the association between the analyzed SNPs and the items of the PSYRATS and Manchester scales. After Bonferroni
correction was applied, only association between SNP rs2253478 and the Poverty of speech was maintained (p corrected=0.038).

With regard to the analyses of potential expansions of trinucleotide tracts of FOXP2, no variation was found in any of these regions in our samples. Only a single deletion of three trinucleotides at the CGG-rich region in intron s1 was identified in heterozygosis in a patient with schizophrenia.

**Methylation analyses**

*FOXP2* has four independent transcriptional start sites that if named using the conventional designations of its exon of initiation would be termed exon s1, exon 1, exon 1b and exon 2. In this work, we have focused on the study of region s1, which is considered a basal transcription start site. Figure 2 is a diagram of the region studied. No differences were found between patients and controls or between brain areas, when the CG1 bisulphite region was analyzed. This region, located upstream of s1 exon was characterized by a general absence of methylation in all the analyzed samples. For the CG2 bisulphite region, located downstream of s1 exon, a higher degree of methylation was found. In addition, subtle differences were found for the parahippocampus gyrus. Within this brain area, the degree of methylation is higher than in the other analyzed areas for both groups, including patients and controls. When right and left parahippocampus gyrus regions were compared, differences in the degree of methylation between patients and controls were obtained (Figure 3). In patients, the degree of methylation is higher in the left hemisphere of the parahippocampus gyrus than in the right one, whereas in controls, methylation is concentrated more in the right hemisphere of the parahippocampus gyrus region.
Interestingly, most of the clones that were analyzed showed similar methylation patterns (Figure 3). A high degree of methylation in the CG2 region was also found in leukocyte samples. In this case, the observed methylation pattern is different from the one obtained from brain samples.

Generally, a high degree of methylation on the promoter region of a gene is correlated with lower RNA expression levels. Therefore, differences in the degree of methylation could result in differential expression of the gene. When comparing the relative expression level of FOXP2 in parahippocampus gyrus between patients and controls higher levels of FOXP2 mRNA expression were obtained in the right hemisphere in patients (Figure 4). This correlates inversely with methylation results, which show a greater degree of methylation in controls than in patients. However, when expression levels from the left and right hemispheres were compared in patients, no correlation was found, since expression is higher in left parahippocampus than in right, as well as the level of methylation. These findings do not support the idea that a high degree of methylation leads to decreased expression of the gene.

**Discussion**

The aim of the present study was to explore the genetic basis of vulnerability to schizophrenia, by examining a positively selected gene, FOXP2, as a candidate gene.

The most important finding of this study is the significant association for SNP rs2253478 and the item of Poverty of speech (P=0.038 after Bonferroni correction). This result relates the FOXP2 gene to one of the characteristic symptoms of schizophrenia, deficits in the language domain [24-26]. When patients with auditory hallucinations were compared with controls significant associations were also found for rs7803667T/rs10447760C/rs923875A/rs2396722C/rs2396753A haplotype, as well as for different allelic frequencies of polymorphisms rs2396753 and
rs17137124. However, differences for both SNPs were lost after Bonferroni correction.

People with schizophrenia are clinically heterogeneous and it has been suggested that probably they have functional/structural deficits or differences in several aspects of the language circuit [12]. This could mean specific language-related circuits are affected in patients with schizophrenia. At this point, our results indicate that in the case of FOXP2, a gene for which an implication in the development of language is well accepted [13,27], the study of language variables in schizophrenia might be more useful than the alternative phenotype. Previously association of FOXP2 and schizophrenia has been reported in Spanish and Korean populations [19,21]. When we considered schizophrenia as a global syndrome (with or without auditory hallucinations), results were not so clear. No significant association was found for any of the SNPs when the different sample groups were compared. In this context, schizophrenia seems to be a phenotype too complex for its genetic analysis and a biological entity not well defined.

In this study, we have tested the role of different structural variations of the FOXP2 gene in relation to schizophrenia vulnerability. These variations included the SNPs discussed previously in this study and potential changes in the number of trinucleotide repeats. Expansions in the number of trinucleotides are frequently associated with neurodegenerative diseases [28]. No variation in the number of glutamines was found in any of our samples. This high stability is concordant with previous studies in controls, individuals with progressive movement disorders, and schizophrenic patients [29,30]. The role of the polyglutamine tracts is unknown in the FOXP2 gene. In fact,
most of the members of the FOX family lack this domain. However, the high invariability suggests that it could be under functional constraints.

In addition to schizophrenia vulnerability due to variations in the sequence of DNA, there must also be epigenetic factors regulating gene expression [31,32]. Epigenetic regulation has been increasingly associated with psychiatric disorders, with examples in depression and addiction [33,34].

In the methylation analysis, we found a higher degree of methylation in the left hemisphere of the parahippocampus gyrus region in patients as compared to controls. Taking those results into account, we would have expected lower gene expression due to repression by methylation. Differences in FOXP2 expression between patients and controls were obtained in the parahippocampus gyrus by quantitative PCR, however, these results were not concordant with methylation analysis. This discrepancy could be explained by the fact that only a stretch of the CpG island located in exon s1 was analyzed for methylation. The promoter region of the FOXP2 gene has not been well defined, and regulation of the gene is more complex than was initially thought (non published personal data, [35]. The finding that expression data show a trend of more expression in patients than in controls, would indicate that a decrease of neural processes controlled by the protein FOXP2, a repressor of transcription, is produced in patients. Hippocampal and parahippocampal volume reduction is one of the most consistent findings in schizophrenia [36]. Moreover, in a meta-analysis of brain volumes in relatives of patients with schizophrenia, hippocampal reduction was the largest difference between relatives and healthy controls [37]. These findings suggest hippocampal volume as a potential end of phenotype for genetic studies in schizophrenia.
This study has some limitations. First, the language skills evaluated in this work include only two items of the Manchester scale. Since the strongest result is related to one of these items, we would recommend a systematic exploration of language variables in schizophrenic patients. In addition, we have used a small sample group in the methylation and expression analyses so further analyses with a larger sample would be necessary in order to confirm our preliminary results. In spite of these limitations this study emphasizes the use of the language related disorder as alternative phenotypes in schizophrenia for genetic studies. Moreover, although the results are not conclusive, this is the first epigenetic study of \textit{FOXP2} in schizophrenia, opening a new way in which this gene could be related to this disorder.

**Conclusions**

In summary, our results do not support the involvement of \textit{FOXP2} gene in the vulnerability to schizophrenia as a global syndrome. Nevertheless it might be involved through its role in language impairment. Epigenetic mechanisms affecting the expression of the gene might be also involved in the developing of this disorder.

**Competing interests**

The authors have declared that no competing interests exist.

**Authors' contributions**

AT participated in the experimental procedure, analysis of results and the draft of the manuscript. AMD participated in the experimental procedure and the draft of the manuscript. MDM, and JS participated in the conception and the design of the study. RF conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors contributed to and have approved the final manuscript.

**Acknowledgements**

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References


Figures

Figure 1 - Structure of FOXP2 gene
Hash marks indicate introns longer than 50 kb. Arrows indicate positions of all single nucleotide polymorphisms (SNPs) analyzed in this study: arrows indicate SNPs polymorphic in our sample. Distances of SNPs to +1 site (5’ end of s1 exon) are shown in brackets.

Figure 2 - CpG dinucleotides in the context of the CpG island located in transcription start site.
The thin black line corresponds to the sequence of DNA. Below this, a single black bar corresponds to the predicted CpG island location. Distances in base pairs to +1 site are included, as well as an arrow indicating the start site of exon s1. Primers for both regions analyzed, CG1 and CG2 are indicated with arrows.
Figure 3 - Bisulfite results for parahippocampus gyrus.
Methylation data are represented as filled circles (methylated CpG) and empty circles (unmethylated CpG) for each bacterial clone obtained. Each row of circles represents the methylation pattern based on the sequence of one cloned PCR product.

Figure 4 - Levels of FOXP2 expression in parahippocampus gyrus brain area.

Tables

Table 1 - Genotypic and allelic frequencies of the SNPs analyzed in patients and controls

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<th>P</th>
<th>Allelic frequencies</th>
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\(a\) Tests in which expected values for more than one class are lower than 5.

\(b\) Corrected p-value (with Bonferroni correction).
### Additional files

**Additional file 1 – Sequences of primers used in the association study, analysis of potential expansions of trinucleotides, methylation analyses and quantitative PCR.**

For SNP rs6961558, one of the primers was modified in order to create a restriction enzyme target depending on the allele in the sequence. Modified nucleotide is shown in grey.

**Additional file 2 – RFLPs conditions.**

**Additional file 3 – Results for Hardy-Weinberg equilibrium test in patients and controls.**

**Additional file 4 – Genotype and allele frequencies of the analyzed SNPs in patients with auditory hallucinations (AH) and controls.**

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</tbody>
</table>

* a tests in which expected values for more than one class are lower than 5.

* b tests in which due to lack of some classes, it was used a table 2x2 instead a 3x2.
* it corresponds to corrected p value (Bonferroni correction).

Additional file 5 – Genotype and allele frequencies of the analyzed SNPs in patients with auditory hallucinations (AH) and patients without AH.

a tests in which expected values for more than one class are lower than 5.

b tests in which expected values for one class are lower than 2.

c tests in which due to lack of some classes, it was used a table 2x2 instead a 3x2.

* it corresponds to corrected p value (Bonferroni correction).
Figure 2

Right Parahippocampus Gyrus

Left Parahippocampus Gyrus

CONTROLS

PATIENTS

Leukocytes
Additional files provided with this submission:

Additional file 1: Additional file 1.pdf, 45K
Additional file 2: Additional file 2.pdf, 51K
http://www.biomedcentral.com/imedia/2144151507353756/supp2.pdf
Additional file 3: Additional file 3.pdf, 24K
Additional file 4: Additional file 4.pdf, 42K
Additional file 5: Additional file 5.pdf, 51K