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Abstract

Catechol-O-methyl transferase (*COMT*) encodes an enzyme involved in the metabolism of dopamine and maps to a commonly deleted region that increases schizophrenia risk. A non-synonymous polymorphism (rs4680) in *COMT* has been previously found to be associated with schizophrenia and results in altered activity levels of COMT. Using a haplotype block-based gene-tagging approach we conducted an association study of seven *COMT* single nucleotide polymorphisms (SNPs) in 160 patients with a DSM-IV diagnosis of schizophrenia and 250 controls in an Australian population. Two polymorphisms including rs4680 and rs165774 were found to be significantly associated with schizophrenia. The rs4680 results in a Val/Met substitution but the strongest association was shown by the novel SNP, rs165774 which may still be functional even though it is located in intron five. Individuals with schizophrenia were more than twice as likely to carry the GG genotype compared to the AA genotype for both the rs165774 and rs4680 SNPs. This association was slightly improved when males were analysed separately possibly indicating a degree of sexual dimorphism. Our results confirm that *COMT* is a good candidate for schizophrenia risk, by replicating the association with rs4680 and identifying a novel SNP association.

Key words: COMT, schizophrenia, genotyping, polymorphism, sexual dimorphism

Introduction

Catechol-O-methyl transference (*COMT*) encodes a catabolic enzyme involved in the degradation of dopamine [1]. *COMT* (as well as 47 other genes) maps to a commonly deleted region on chromosome 22q11 [2]. When deleted, the region results in a marked increase in susceptibility to schizophrenia [3-5] making *COMT* a strong candidate gene for schizophrenia. In addition, a polymorphism that results in a Val/Met substitution at codon 158 (rs4680) results in altered activity levels of COMT [6]. Homozygotes for the Met allele have reduced COMT enzyme activity compared to homozygotes for the Val allele [7,8]. While heterozygotes have intermediate enzyme activity indicating that a partially dominant mode of inheritance is operating [9]. One study suggests that the high activity Val allele may increase schizophrenia risk by increasing catabolism of dopamine in the prefrontal cortex [10]. The only other functional polymorphism that has been identified in *COMT* to be associated with altered enzyme activity is an Ala/Ser substitution at codon 72 (rs6267) [11]. This polymorphism has only been found to be associated with schizophrenia and altered enzyme activity in Asian populations [11,12].

Through linkage and association studies, *COMT* has been extensively investigated and association with schizophrenia has been established particularly for the rs4680 polymorphism [10,13-18]. However meta-analyses of *COMT* have not confirmed these associations. A meta-analysis of case-control studies did not find association with the Val/Met polymorphism but association was observed in family-based linkage studies of European descent [19]. In a further meta-analysis study, association with Val/Met polymorphism and schizophrenia was found but when studies were excluded where controls departed significantly from Hardy-Weinberg equilibrium, association was no longer observed [20]. A recent Japanese meta-analysis did not report association of the Val/Met or Ala/Ser polymorphisms [21].

To identify novel polymorphism associations and confirm previous association studies, seven SNPs in *COMT* were selected that mark haplotype blocks, covering the entire *COMT* gene. These seven SNPs were then genotyped in an Australian population to investigate association with schizophrenia.

Materials and methods

Subjects

The study included 160 Caucasian schizophrenia patients and 250 unrelated Caucasian controls. Subjects were aged between 18 and 65 years. Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) diagnosis of schizophrenia was confirmed by at least two independent psychiatrists. These patients had never been diagnosed with other psychiatric disorders, including schizoaffective disorder, major depressive episode with psychotic features, or bipolar disorder. No patients were treated with antidepressants, anxiolytic agents, or mood-stabilizing psychotropic medications and all were maintained on a constant dose of antipsychotic medication for a minimum of three weeks. There were 22 females and 138 males in the group diagnosed with schizophrenia with a mean age of 36.2 years (s.d. $\pm$ 12.1 years). The mean age of patients at first diagnosis (onset of psychotic symptoms) was 23.4 years (s.d. $\pm$ 7.47 years). They were being treated at the Royal Brisbane and Women's Hospital, The Park Psychiatric Unit, and the Valley Community Psychiatric Centre. The clinical sample was composed of 69 inpatients and 91 outpatients. All patients were administered the Positive and Negative Symptom Scale (PANSS) [22] to assess severity of psychotic features. The PANSS total mean score was 45.013, SD. 13.646. A history of psychological distress, indicated by past suicide attempt, was reported by 74 patients. A total of 121 patients were able to provide information on psychiatric illness among first-degree

relatives and 82 of these patients (68%) reported a positive family history of schizophrenia. This clinical sample was a group who had lived with schizophrenia for an average of 13 years since diagnosis continued to experience positive and negative symptoms despite treatment with antipsychotic medication. Mean length of illness was 13.39 years, SD 10.91. In addition the schizophrenia group contained a high proportion of individuals with a relatively severe history and/or a familial risk for psychosis.

The control group consisted of 102 females and 148 males, with a mean age of 36.8 years (s.d. $\pm$ 12.8 years). The control group consisted mostly of medical and nursing staff recruited through hospitals, and university students and academic staff. Formal screening for schizophrenia or other psychological disorders was not undertaken in the control population. As such, the controls represent an unselected control group.

Each patient gave written informed consent. Ethics approval was obtained from all institutions involved.

Selection of SNPs

Using the International HapMap Project (HapMap) phase II [23], five tag-SNPs in *COMT* with a minor allele frequency ≥ 0.2 were selected. Tag-SNPs were identified using the pair-wise option of Tagger with a threshold of $r^2 > 0.8$. Two non-synonymous SNPs reported in The National Center for Biotechnology Information (NCBI) SNP database (dbSNP) were also chosen.

Genotyping

Oragene kits were used to extract DNA from saliva samples. Samples were genotyped using a homogeneous MassEXTEND (hME) Sequenom assay performed by the Australian Genome Research Facility. The hME assay is based on the annealing of an oligonucleotide primer (hME primer) adjacent to the SNP of interest. The addition of a DNA polymerase along with a mixture of terminator nucleotides allows extension of the hME primer through the polymorphic site and generates allele-specific extension products, each having a unique molecular mass. The resultant masses of the extension products are then analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and a genotype is assigned in real time. The hME assay was performed in multiplex with up to 36 reactions in a single well.

The genotyping of several other SNPs were independently verified by other methods such as real-time PCR with excellent correspondence (the COMT SNPs were not independently verified). The genotyping fail rate for each SNP was: rs165774 3%; rs4680 8%; rs737866 5%; rs740603 6%; rs6267 4%; rs174675 4%.

Statistical Analysis

A Pearson's chi-squared test was performed to identify statistical associations between alleles/genotype and schizophrenia status. Odds ratios (OR) were also calculated. Tests were performed on both genotype and allele data. Statistical tests were performed using the COMPARE2 program from the WinPepi suite of epidemiology programs [24] and SPSS version 16.0. Hardy-Weinberg equilibrium (HWE) was computed using Utility Programs for Analysis of Genetic Linkage [25]. The analysis of genotypes under a recessive model involved pooling the low-risk homozygotes and the heterozygotes and comparing frequencies with the high-risk homozygotes, i.e. OR > 1. Correction for multiple testing was conducted

using the Benjamini-Hochberg method [26]. Linkage disequilibrium and haplotype estimations were calculated using JLIN version 1.6.0 [27]

Results

In order to identify genetic association with schizophrenia, polymorphisms in the *COMT* gene were genotyped after selection using a haplotype block-based gene-tagging approach. A total of five tag-SNPs and 2 non-synonymous SNPs from NCBI were genotyped in a sample of 160 schizophrenia patients and 250 controls. One of the non-synonymous SNPs, rs6267 (Ala72Ser) was excluded from the final analysis after genotyping revealed no heterogeneity in our patient or control populations. Three of the remaining six SNPs analysed were found to be significantly associated with schizophrenia at the allele level (Table 1). These included two tag-SNPs (rs165774 and rs4646316) and one non-synonymous SNP (rs4680). Both the rs165774 and rs4680 associations survived after correction for multiple testing but the rs4646316 association did not (corrected α-values varied between 0.025 and 0.008, for the two surviving significant SNPs). These three SNPs were selected for further analysis at the genotype level (Table 2). Only two of the SNPs, rs165774 and rs4680 remained significant at the genotype level. However, all three SNPS were significant at the genotype level when analysed using the Mantel-Haenszel test for trend (partially dominant model) and all survived correction for multiple testing (corrected α-values varied between 0.05 and 0.017). Interestingly, the strongest association at either the allele or genotype level was displayed by the intronic tag-SNP, rs165774.

In the control and schizophrenia samples strong linkage disequilibrium (LD) was observed between all three pairs of loci (*D'* from 0.71-1). The tightest linkage (*D'* = 1) was observed in the schizophrenia samples between the rs4646316 and rs165774 polymorphisms, which were

separated in the gene sequence by only 429 bp. The estimation of haplotype frequency for the three loci by an expectation-maximization algorithm revealed no statistically significant differences between the schizophrenia and control samples.

Genotype frequencies indicated that all polymorphisms were in HWE in both case and control samples.

Examination of the genotype odds ratios (OR) of the rs165774 and rs4680 SNPs suggests that a partially dominant mode of inheritance is operating for schizophrenia, i.e. the heterozygous OR was intermediate between the associated and non-associated genotypes (Table 2).

In order to evaluate whether there was any sexual dimorphism in the population, the male schizophrenia patients were analysed separately from the female patients. At the allele level, the same three SNPS showed association with disease with slightly improved p-values (rs165774, Pearson $\chi^2 = 8.929$, $p = 0.03$; rs4646316, Pearson $\chi^2 = 4.489$, $p = 0.034$; rs4680, Pearson $\chi^2 = 6.403$, $p = 0.011$). At the genotype level, rs165774 showed association with schizophrenia with a slightly improved p-value of 0.018 (Pearson $\chi^2 = 7.985$) and rs4680 had the same p-value of 0.044 (Pearson $\chi^2 = 6.254$). The male schizophrenia samples remained in HWE. The remaining female cases were too small in number to analyse meaningfully.

Positive and negative symptom scale ratings and age of onset were also collected for each schizophrenia patient and correlated with genotype for each polymorphism. Analysis of variance was performed on genotype versus PANSS positive rating scale, PANSS negative rating scale, PANSS general scale and PANSS total scale as well as onset age. No significant associations were found.

Discussion

Analysis of five tag-SNPs and two non-synonymous SNPs in *COMT* revealed associations

with schizophrenia at the genotype and allele level for two polymorphisms (rs165774 and

rs4680) in an Australian schizophrenia cohort. The rs4646316 SNP was also found to be

significant but only at the allele level and the association did not hold up after correction for

multiple testing. Given our results and the fact that two other studies failed to identify an

association with schizophrenia [28,29], rs4646316 is unlikely to be involved in susceptibility

to schizophrenia. The rs165774 has been analysed previously but an association with

schizophrenia has not been identified [28-30]. We may have observed an association with

schizophrenia and rs165774 because of the high heritability seen in our patient cohort. In our

study 68% of patients reported a positive family history of schizophrenia. Our clinical

sample was a group who had lived with schizophrenia for an average of 13 years since

diagnosis and continued to experience positive and negative symptoms despite treatment with

antipsychotic medication. Therefore our samples consisted of a high proportion of

individuals with a relatively severe history and familiar risk for schizophrenia.

Previous results for the Val/Met (rs4680) SNP are more controversial with a number of

positive associations [10,13-18,31-33] but also many negative associations with

schizophrenia identified [11,18,28,29,34]. Our findings as well as numerous others support

the Val/Met polymorphism as a risk factor for schizophrenia susceptibility. Our results also

support a partially dominant mode of inheritance for the rs4680 SNP which is consistent with

an earlier study that found individuals who were heterozygous for rs4680 had intermediate COMT

enzyme activity compared to homozygotes [9]. Failure of other studies to replicate the

association of rs4680 with schizophrenia could be the result of clinical heterogeneity of the cases.

In a Korean population an Ala72Ser polymorphism (rs6267) was found to be associated with schizophrenia but the Val/Met polymorphism was not associated [11]. The rs6267 polymorphism displayed no heterogeneity in our Caucasian study suggesting that it is a racial marker. In a Chinese population the rs4633 SNP (not analysed by us) was found to be significantly associated with schizophrenia but not rs4680 [30]. The Chinese study concluded that ethnic background might be one of the major reasons for the controversial results. The SNP rs4633 has been shown to be in complete linkage disequilibrium with rs4680 in a European study [35] but the Chinese study did not observe LD in their population. They concluded that rs4633 rather than rs4680 is likely to be the susceptibility SNP for schizophrenia in a Chinese population [30].

Some evidence suggests that association between *COMT* and schizophrenia is different between men and women [33,36]. In addition, COMT enzyme activity in the prefrontal cortex is higher in men than women [6]. To address some of these differences we analysed males separately for *COMT* association with schizophrenia. Despite reducing the number of samples in the analysis, the significance of association between all three SNPs and schizophrenia was not only maintained, it was slightly improved when only males were analysed. This is consistent with at least a degree of sexual dimorphism in genetic susceptibility to schizophrenia in males. This is perhaps not surprising considering the differences in schizophrenia environmental risk factors for males and females and the possibility that they might be acting on different susceptibility genes. A recent study found association between the Val allele of the rs4680 SNP and schizophrenia in males but not females in a Spanish population [36]. They also observed a strong deviation from Hardy-

Weinberg equilibrium in their male population. In contrast, an earlier Turkish study found association with the rs4680 SNP and schizophrenia to be stronger in females [37].

The rs165774 SNP displayed stronger association than rs4680 at both the allele and genotype levels but it is in tight LD with the non-synonymous SNP rs4680. Despite the fact that it is an intronic tag-SNP, it is still possible that rs165774 is functional or it is possible that it is in linkage disequilibrium with another nearby functional SNP such as rs737865 or rs165599 [33,38].

Conclusions

In order to obtain optimal clinical homogeneity in our schizophrenia group, the clinical evaluation of patients from the same catchment area was performed by senior psychiatrists and diagnosis of schizophrenia (DSM-IV) was confirmed by at least two independent psychiatrists. Control samples collected from the same catchment area as the cases were not screened for psychiatric conditions. However not screening controls is unlikely to bias results as screening would only strengthen the associations observed between cases and controls. Although cases and controls were screened for Caucasian ethnicity, population stratification can't be ruled out. However, our results for the rs4680 SNP have been confirmed by numerous other groups in Caucasian populations. The other significant SNP, rs165774 ideally needs to be confirmed in other Caucasians groups as well as defined ethnic groups. While our association study does not have an extremely large number of cases and controls, the numbers are still strong and we obviously had sufficient power to detect association. Our numbers may not be sufficiently large enough to exclude association with the rs4646316 SNP and the *p*-value was approaching significance at the genotype level and was significant at the allele level.

In summary we have confirmed association of the Val/Met polymorphism with schizophrenia in our Australian population and have demonstrated that rs165774 could also increase schizophrenia risk.

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Author Contributions

JV: Involved in conception and design, analysed and interpreted data, drafted article, critically revised article and approved final version of article.

CS: Involved in conception and design and approved final version.

IH: Involved in conception and design and approved final version of article.

BL: Involved in conception and design and approved final version.

RY: Involved in conception and design, critically revised article and approved final version.

CM: Involved in conception and design, analysed and interpreted data, critically revised article and approved final version.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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