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Genes involved in pruning and inflammation are enriched in a large mega-sample of patients affected by Schizophrenia and Bipolar Disorder and controls

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Abstract

A molecular pathway analysis has been performed in order to complement previous genetic investigations on Schizophrenia. 4,486 Schizophrenic patients and 4,477 controls served as the investigation sample. 3,521 Bipolar patients and 3,195 controls served as replication sample. A molecular pathway associated with the neuronal pruning activity was found to be enriched in subjects with Schizophrenia compared to controls. HLA-C and HLA-DRA had more SNPs associated with both Schizophrenia and Bipolar Disorder than expected by chance.

Keywords

Schizophrenia; Pathway Analysis; Pruning

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Conflict of Interest

The Authors declare that they have no conflicts of interest.

Contributors

All authors contributed to and have approved the final manuscript. In particular: Authors Antonio Drago, Concetta Crisafulli and Marco Calabrò designed the study. Author Marco Calabrò managed the literature searches and the analyses and wrote the first draft of the manuscript. Authors Antonio Drago and Concetta Crisafulli managed the Statistical analyses and wrote the protocol. Author Alessandro Serretti supervised the study.

Additional information about methods used in this paper may be found on supplementary material.

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1. Introduction

Schizophrenia (SCZ) is a complex disorder ranking at the top ten causes of long-term disability worldwide (Beers, 2004). SCZ runs in families (Sullivan et al., 2003) and a number of independent investigations confirmed that genetic variations may be associated with SCZ (Hosak et al., 2012; Li et al., 2010; Rujescu et al., 2009; Shi et al., 2009; Stefansson et al., 2009; Walker et al., 2010; Williams et al., 2011). However, the complete picture of its genetic background remains elusive (O'Connell et al., 2011). Separating the impact of common genetic variants with a small effect towards SCZ from the false positive associations (Cantor et al., 2010) may prove to be difficult. A molecular pathway analysis may help by taking into account several variations harbored by different genes per time, clustered in consistent molecular groups. This perspective may successfully capture the signal of a number of genetic variations with a small single effect. This approach is consistent with the polygenic nature of SCZ and it may reveal some aspects of the molecular basis of the disorder. We undertook a genome-wide molecular pathway analysis to test the hypothesis that the genetic variations associated with SCZ segregate in specific molecular pathways within the genome. The study was conducted in a large sample of subjects with SCZ and controls (n=8963) and then replicated in a large sample (n=6716) of subjects with Bipolar Disorder (BD). The shared genetic background of the two disorders (Cross-Disorder Group of the Psychiatric Genomics, 2013; Ivleva et al., 2010) is the rationale of this replication.

2. Methods

2.1. Pathway analysis: a tool for the study of polygenic disorders

A large pool of genes harboring variations with little impact on the risk for SCZ may prove difficult to be identified in common genetic association approaches. An entire biological process rather than single mutations in one or more random genes were then tested in the present study, in order to cluster variations with small impact on SCZ in consistent molecular pathways, so that the genetic effect on the risk for SCZ may be more easily recognized. The alteration of a biological process may be caused by a single mutation at the level of one of its nodal point or by multiple mutations with little effects on different sets of genes with a more marginal role in the pathway. This could explain the considerable amount of genes found associated with Schizophrenia and Bipolar Disorder, along with the inconsistent findings in literature. The pathway analysis stresses this hypothesis. Group of genes were then clustered in molecular networks. The resulting Network as a whole was then tested as a genetic risk factor for SCZ, and the finding replicated in BP subjects.

2.2. Flowchart of the study

The study was developed as shown in the following diagram (Fig. 1).

The key points of the study are shown in step 3 and 4 - the genes extracted from literature were used to create a molecular network, that was then enriched and tested for association with SCZ; and point 5b - where the genes that held the highest number of variations associated with SCZ (at least ten times the expected number by chance) were clustered in highly significant clusters, enriched and re-submitted to the permutation analyses.

2.3. Dataset

Data of the SCZ and BD samples under investigation was retrieved from public NIHM databases. Table S1 in supplementary section reports the characteristics of the samples.

2.4. Genetic quality control

Genetic quality control included relatedness testing and principal components analyses. Identical subjects ($\pi\text{-hat} > 0.9$) were identified and one was included after random choice. Relatives ($\pi\text{-hat} > 0.2$) were excluded as well, by randomly choosing one representative. Principal component estimation was done with the non-related subset of individuals. The population structure and the study of origin were chosen as covariates along with the gender.

2.5. Imputation

Imputation was run for the genes that belong to the pathway under analysis. The data of HapMap 1000 genomes database on CEU population served for the imputation. IMPUTE2 (Howie et al., 2009) was used to impute the missing genetic information.

2.6. Pathway enrichment analysis

The genomic sections that contain the genes included in the selected networks were identified through PLINK annotation. These sections were imputed, checked for quality ($\text{info} > 0.9$) and pruned ($r^2 > 0.2$) and their association with the phenotype (schizophrenic patients vs controls or bipolar patients vs controls) tested (see supplementary materials). The prevalence of variations significantly ($p < 0.01$) associated with the outcome in the pathway under analysis and randomly generated pathways were tested for a significant different distribution (Fisher exact test). Fisher test identifies the significant clustering of SNPs associated with the phenotype in the original pathway by comparing the distribution of SNPs in two pathways, the original and a random generated one. This operation was repeated 106 times (permutation) selecting different groups of random SNPs as a control group. 106 Fisher Tests were then conducted and the permuted p was extracted according to Phipson and Smyth (2010).

2.7. Toolset for the analysis

Cytoscape (Saito et al., 2012) and the related add-on GeneMania (Warde-Farley et al., 2010; Zuberi et al., 2013) were used to analyze and enrich the set of genes initially chosen from literature. The add-on MCode served to identify a number of 8 sub-clusters. Executables (BASH language) were written for operationalize the analyses (available upon request). R (R Core Team, 2014), PLINK (International Schizophrenia et al., 2009; Purcell et al., 2007), SNPTESTv2.3.0 (Marchini et al., 2007) and IMPUTE2 were part of the executables and served for the analyses.

2.8. Testing the hypothesis

This analysis was conducted in a sample of GWAS that partially overlapped those used for the selection of the candidate pathways. Apparently, this is like searching a confirmation of a finding starting from the same specimen that provided that result. In this case though, we used a method for analyzing the data that differs from the methods used in the GWAS that

provided the results from which we started. Thus, the replication of a set of findings by using this new method can be used as a validation of the method itself. In particular, we expected to validate the results from the larger GWAS published so far that used a sample partially overlapping the one investigated here. If confirmed, this finding would pave the way to the use of this method in new independent samples. Moreover, our analysis differs from previous publications in that we analyzed a mega-sample that derived from the merging of individual samples.

3. Results

Lambda values did not show evidence of major population stratification factors neither in SCZ - Lambda of 1.04 (Standard Error[SE] = 4.15e-05; Lambda1000 = 1.02; SE = 1.50e-05); nor in the BD - Lambda = 1.23 (SE = 4.42e-05; Lambda1000 = 1.02; SE = 4.19e-06). The observed inflation factor in the total sample was interpreted as indicative of a large number of weakly associated SNPs consistent with the disease's polygenic inheritance (Schizophrenia Psychiatric Genome-Wide Association Study, 2011).

The network obtained in step 3 of the flowchart (Fig. 1) was significantly associated with SCZ (permutated(10^6) p value = 0.037).

From the clusters elaborated in point 5b of the flowchart (Fig. 1), the one associated with the activity of HLA-DRA and HLA-C were found to have more variations associated with the phenotype than expected by chance. These systems are involved in pruning in the CNS (Faissner et al., 2010; Stephan et al., 2012). HLA-DRA was found to harbor more variations than expected by chance in the samples: 19 out of 108 in the SCZ sample (17% versus the expected 1%), and 2 out of 89 in the BD sample (2% versus the expected 1%). HLA-C was also found to harbor more significant variations than expected by chance: 18 out of 73 in the SCZ sample (24% versus the expected 1%), and 2 out of 62 in the BD sample (3% versus the expected 1%). Rs9268853 and rs9268858 were significantly associated with the phenotypes in both samples. The minor allele was more represented in cases than in controls in both samples. The additional pathways investigated showed no significant associations.

4. Discussion

Consistently with previous findings in literature (Gilvarry et al., 1996; Muller et al., 1999a; Rothermundt et al., 2001; Strous and Shoenfeld, 2006), we found evidence of an immune/autoimmune component in SCZ.

The immune hypothesis of SCZ has gained credibility thanks to studies performed in recent years (Krause et al., 2012; Saetre et al., 2007; Sequeira et al., 2012) and especially after the detection of novel functions of the immune molecules in the brain and bidirectional cross-talk between the Immune System(IS) and the Central Nervous System (CNS) (Tian et al., 2012).

Several studies (Axelsson et al., 1982; Karanikas, 2011; Muller et al., 1999b; Schwarz et al., 2000) that have reported an impaired blood-brain barrier in SCZ subjects, suggest that

psychotic phenotype may be caused by degenerative immune-processes, similarly to Multiple Sclerosis (MS) (Amital and Shoefeld, 1993; Ruutinen et al., 1981).

Moreover, i) immune-inflammatory genes are up-regulated in the CNS (Saetre et al., 2007); ii) immune-inflammatory events have a critical role in the remodeling of glutamatergic synapses (Fourgeaud and Boulanger, 2010) whose disruption increase the risk for SCZ; iii) Immune System modulates the synaptic function (Schmitt et al., 2011).

Finally, closer to the interest of this paper, rs9268853 and rs9268858, two of the SNPs we found to be significantly associated with both SCZ and BD, are also associated with MS (NCBI dataset).

Recent studies indicate an increased microgliosis-astrogliosis ratio in SCZ (Wierzbica-Bobrowicz et al., 2005), pointing that microglia cells may play an active role in the molecular processes involved with this disease.

Activated Microglia elements can act as antigen-presenting cells in CNS. These cells normally express low levels of MHCII proteins but pathological or inflammatory conditions up-regulate their expression (Kreutzberg, 1996; O'Keefe et al., 2002), possibly altering Microglia functions.

Considering the role of Microglia in neurodevelopment (Alliot et al., 1999) and in neuronal remodeling mechanics, including apoptosis, axon remodeling and synaptic pruning (Pont-Lezica et al., 2011), this activation could cause serious consequences in the shape and functions of neuronal nets. This scenario is consistent with typical deficits of SCZ and is also consistent with the age at onset of the disease, early adulthood, which is characterized by an intense pruning activity (Paus et al., 2008). This mechanism is also consistent with a recent hypothesis that underline the relevance of neuronal pruning in the development both SCZ and BD (Strakowski et al., 2012).

4.1. BD replication

SCZ and BD may share a relevant part of their genetic background (Shao and Vawter, 2008). Most association studies linking the MHC region with BD involve the classical HLA genes. Modrego and Ferrández (2000) observed an increased frequency of HLA-DR2 and HLA-DR3 in patients with BD. In 2009, the International Schizophrenia Consortium detected a hotspot on chromosome 6p22, in which are located HLA genes, with a highly significant association with both SCZ and BD (International Schizophrenia et al., 2009). Consistently with this set of evidence, we found that the same genes (HLA-C and HLA-DRA) harbored more variations associated with the disorder than expected by chance in both the SCZ and BD samples.

4.2. Limitations

The analysis is based on the knowledge of the variants associated with the disease, and the enrichment was conducted starting from the genes known to be associated with SCZ. This means that this approach will explore only pathways linked with the first selection of genes. As a result, this method cannot provide new unexpected genes associated with SCZ, even

though it is designed to enlarge the list of possible candidates in consistent metabolic pathways. Further, it has to be noted that our positive findings in HLA region may be influenced by the presence of LD pairs. HLA region is known for having a very complex LD structure that is not removed by normal pruning. We tried to limit this bias using a very stringent cutoff, however some LD pairs may have survived. Our method has proven to provide results consistent with the previous investigations and more classic GWAS approaches but it adds relevant information (Debnath et al., 2013; Drago et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics, 2014). Although further tests are needed to completely validate this approach, the consistency we found between the data obtained with this method compared with more classic analyses, suggests this strategy as a possible useful complement in the study of the genetics of psychiatric disorders in independent samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A biomolecular network related to schizophrenia was identified from literature
- The network was clustered in sub-pathways. Each pathway was independently analyzed
- “Inflammation and neuronal pruning” pathway was associated with psychotic phenotype

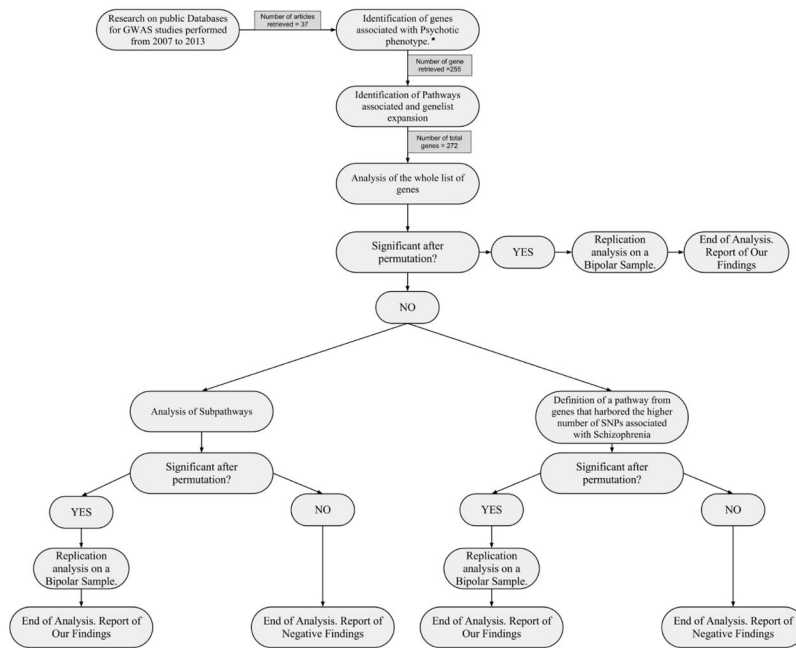


Fig 1. In the above figure is presented the flowchart of our analyses. Each node indicate a different stage of our work and the possible development of the analyses according to the data obtained at each stage.

*Psychotic phenotype: psychotic symptoms included any positive or negative schizophrenic symptoms in absence of organic disorder