Journal of Molecular Neuroscience (2018) 64:273–286
https://doi.org/10.1007/s12031-017-1016-8

Hot Genes in Schizophrenia: How Clinical Datasets Could Help to Refine their Role

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Received: 2 October 2017 / Accepted: 12 December 2017 / Published online: 19 December 2017

Abstract

We investigated the effect of a set of SNPs within 5 genes identified by GWASs as possible risk genes for schizophrenia (SCZ) in two independent samples, comprising 176 SCZ patients and 326 controls of Korean origin and 83 SCZ patients and 194 controls of Italian origin. The PANSS was used to assess psychopathology severity and antipsychotic response (AR). Several clinical features were assessed at recruitment. In the Korean sample, the SP4 gene haplotype rs2282888-rs2237304-rs10272006-rs12673091 ($p = 0.02$) was associated with SCZ. In the Italian sample, PPP3CC rs11780915 (genotypic: $p = 0.006$; allelic: $p = 0.001$) and rs2249098 (genotypic: $p = 0.0004$; allelic: $p = 0.00006$) were associated with SCZ, as well as the PPP3CC rs11780915-rs10108011-rs2249098 and the ZNF804A rs7603001-rs1344706 haplotypes ($p = 0.03$ and $p = 0.02$). Several RORA variants were associated with AR in both the samples, although only the haplotype rs1020729-rs1871858 in the Korean sample survived to the statistical correction ($p = 0.01$). Exploratory analyses suggested that: (1) PPP3CC, ST8SIA2, and SP4 genes may modulate psychotic symptoms, and (2) RORA and ZNF804A genes may influence AR. Our results partially support a role for these genes in SCZ and AR. Analyses in well phenotyped samples may help to refine the role of the genes identified by GWASs.

Keywords Schizophrenia · Genetic · Pharmacogenetics · PPP3CC · RORA · ST8SIA2 · SP4 · ZNF804A

Introduction

Schizophrenia (SCZ) is a highly heritable disorder which affects about 1% of the population, and it ranks among the top 10 causes of disability worldwide (Organization WH, 2008; Tandon et al. 2008). Although environmental factors play a relevant role in the etiology of SCZ, the high heritability points to a major role for inherited genetic variants (Schlossberg et al. 2010; Sullivan et al. 2003). Risk variants for SCZ are thought to range from common to extremely rare (Sullivan et al. 2012); this makes their detection more difficult. Consequently, candidate gene studies performed in the last decades failed to find consistent results about SCZ genetics background (Chen et al. 2015). Similarly, a genetic contribution for antipsychotic response (AR) has been suggested (Brandl et al. 2014). Nonetheless, also pharmacogenetics studies on AR were mainly inconclusive so far (Brandl et al. 2014), despite AR has been thought to be closer to genetic factors compared to the disorder itself and thus it has been largely used as intermediate phenotype in the last decades. A

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12031-017-1016-8) contains supplementary material, which is available to authorized users.

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significant step forward in SCZ genetics and pharmacogenetics fields was reached with the spreading of genome wide association studies (GWASs), which analyzed the entire genome without any a priori hypothesis. In the last years, GWASs finally provided consistent results about SCZ genetics background (2014). However, the role of the genes identified in SCZ is still largely unknown and GWASs samples (which require > 10,000 patients to be reliable) are rather inadequate to clarify their functions because they lack of detailed clinical information about the subjects included.

Taking into account these considerations, in the present study, we aimed to investigate the effect of five genes discovered by GWASs in two independent, well-phenotyped samples. Beyond the standard case-control and pharmacogenetics analyses, we performed a number of exploratory analyses in order to elucidate the specific role of these genes in SCZ pathophysiology. Although this approach increased the number of analyses, the strong a priori genes selection should guarantee versus false positive findings. Furthermore, the use of two independent samples together with a critical clinical interpretation of findings may further reduce the possibility of false positive results. However, the only aim of our exploratory analyses was to suggest further investigations; thus, they should be considered carefully.

On the basis of recent literature data from both genetics (Ripke et al. 2014) and pharmacogenetics studies, we selected the following five genes for investigation: protein phosphatase 3, catalytic subunit, gamma isozyme (PPP3CC) (Kyogoku et al. 2011; Sacchetti et al. 2013; Wockner et al. 2014), RAR-related orphan receptor A (RORA) (Adkins et al. 2010; Devanna and Vernes 2014; Garriock et al. 2010; Le-Niculescu et al. 2009), Sp4 transcription factor (SP4) (Fabbri et al. 2015; Tam et al. 2010), ST8 Alpha-N-Acetyl-neuraminide alpha-2,8-sialyltransferase 2 (ST8SIA2) (Lee et al. 2011; Vazza et al. 2007), and zinc finger protein 804A (ZNF804A) (ISGC, WTCCC2 2012; Lee et al. 2012; Sun et al. 2015). These genes were selected among the ones identified by GWAS also because they are involved in molecular processes which are thought to play a relevant role in the etiopathogenesis of SCZ and other neuropsychiatric disorders. More in detail, they were selected because of (1) their involvement in neuroplasticity processes (PPP3CC (Xia and Storm 2005) and ST8SIA2), (2) their effects on genes transcription (SP4 (Mao et al. 2007; Zhou et al. 2007) and ZNF804A (Esslinger et al. 2011; Lencz et al. 2010)), and (3) their involvement in circadian rhythms (RORA (Buhr and Takahashi 2013)). Within these genes, the genetic variants investigated were selected on the basis of previous literature data or because they were tag SNPs (http://hapmap.ncbi.nlm.nih.gov/) with a prevalence of at least 5% in the population under investigation (for details, see Table 2).

Methods

Design of the Study

Korean Sample

The Korean sample included 176 SCZ Korean in-patients and 326 Korean psychiatrically healthy subjects, which were recruited at the Department of Psychiatry of the Catholic University of Korea, College of Medicine. For detailed description of the sample and the assessment performed, please refer to previous studies (Porcelli et al. 2015). Briefly, all patients were admitted to the inpatient unit for psychotic relapse and they were discharged when clinical conditions allowed to continue treatment as outpatients. Inclusion criteria were a diagnosis of SCZ according to the Diagnostic and Statistical Manual of Mental Disorders IV Edition-Text Revised (DSM-IV-TR) criteria (Association 2000), as assessed by the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Sheehan et al. 1998). Exclusion criteria were current severe or unstable medical and neurological conditions, current treatment with a long-acting antipsychotic, concomitant alcohol and substance abuse disorders, and to be of ethnicities other than Korean. Healthy subjects were controls who underwent the same assessment of psychiatric patients to exclude possible psychiatric disorders.

All patients admitted to the hospital were assessed for the severity of illness at baseline and at discharge through the administration of the Positive and Negative Symptoms Scale (PANSS) (Kay et al. 1987). Scorers were trained with good inter-rater reliability ($k = 0.8$). Additionally, several clinical and demographic variables were recorded. The study protocol was approved by the institutional review board (approval number HC10TISI0031). All subjects provided written informed consent before participating to the study.

Italian Sample

The present study was conducted in a naturalistic setting. Eighty-three SCZ patients were enrolled into the study when admitted at the Psychiatric inpatient Unit, Department of Psychiatry, University of Bologna, Italy. Inclusion criteria were age from 18 to 75 and a diagnosis of schizophrenia according to the DSM-IV-TR criteria and confirmed by M.I.N.I. Patients were included if they needed to start or to change antipsychotic treatment for a psychotic relapse. They were treated according to the current clinical practice, without any limitation concerning the kind of antipsychotic or the dosage. Exclusion criteria were the presence of severe medical conditions or moderate to severe dementia (Mini Mental State Examination score < 20). Clinical and demographic characteristics of patients were assessed at recruitment. The PANSS was administered at baseline and every 3 days until the

discharge by trained medical staff. Scorers were trained with good inter-rater reliability ($k = 0.8$). For the pharmacogenetic analysis, we took into account the repeated PANSS scores until day nine (four evaluations). The control sample comprised 194 healthy subjects and it was extracted from a bigger sample already described elsewhere (De Ronchi et al. 2005; Forlani et al. 2014). Written informed consent was obtained for each subject recruited.

The study protocols were approved by the local Ethical Committees and they have been performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki.

Genotyping

Korean Sample

Genomic DNA of Korean samples was purified from whole blood using the QIAamp DNA Blood Midi Kit (Qiagen, CA, USA). Genotyping was performed through a pyrosequencer (Biotage AB, Sweden). PCR primers (sequences available on request) (Bioneer, Daejeon, Korea) and sequencing primers (Bioneer) used for the pyrosequencing assay were designed by using the Pyrosequencing Assay Design Software v.1 (Biotage), with one biotinylated primer for each pair. In the Korean sample, 4 SNPs within the PPP3CC gene, 27 SNPs within the RORA gene, 5 SNPs within the SP4 gene, 12 SNPs within the ST8SIA2 gene, and 7 SNPs within the ZNF804A gene were analyzed (see Supplementary Figs. 1 and 2 for detail).

Italian Sample

Genomic DNA of Italian samples was extracted from blood sample thorough an automated magnetic beads-based nucleic acids extractor (Maxwell, Promega, Madison, WI). Standard protocols were used for the above procedure (Maxwell, Promega, Madison, WI). Presence of the investigated SNPs within each sample was checked by a multiplex Sequenom MassArray platform. (Sequenom Inc., CA, USA). Sequenom's MassARRAY Designer software was used to design PCR and extension primers (sequences available on request) for each investigated SNP. In the Italian sample, three SNPs within the PPP3CC gene, four SNPs within the RORA gene, three SNPs within the SP4 gene, eight SNPs within the ST8SIA2 gene, and two SNPs within ZNF804A gene were analyzed (see Supplementary Figs. 1 and 2 for detail). At least two readings were performed for each sample. Samples showing ambiguous alleles were discarded if they showed the same features on repeated genotyping. The final call rate was more than 99.6% for each SNP. All analyses were executed by personnel blind to diagnostic and clinical status of the subjects.

Statistical Analysis

Traditional statistical analyses were performed using "Statistica" package (StatSoft I. STATISTICA 7.0 per Windows: StatSoft, Inc. 1984–2004, Tulsa, Oklahoma, USA) while test for association using multi-marker haplotypes were performed using the statistic environment "R cran," package "haplo.score" (http://cran.r-project.org/). Differences in the allelic and genotype frequencies were calculated using the χ^2 statistics. Repeated measure analysis of variance (ANOVA) was used to investigate the AR. Haploview 4.2 (Haploview 4.2; Bioinformatics. 2005. Daly Lab at the Broad Institute Cambridge, Massachusetts 02141, USA) was used to generate a linkage disequilibrium (LD) map and to test for Hardy-Weinberg equilibrium (HWE) (Barrett 2009). The Haploview 4.2 software automatically generates the haplotypes to be investigated on the basis of the algorithm developed by Gabriel et al. (2002). Further, other haplotypes were selected by authors on the basis of strong LD ($D' > 85$), proximity of SNPs, and prevalence more than 1%. Permutations ($n = 100{,}000$) were performed to estimate the global significance of the positive results obtained.

The main outcome measures were: (1) differences among genotypic and allelic frequencies between cases and controls and (2) influence of the SNPs investigated on AR, as measured by PANSS total scale and analyzed through repeated measure ANOVA. In case of positive findings, the following clinical variables were added as covariates: sex, age, age of onset, psychiatric family history, previous suicide attempts, duration of the illness, and the antipsychotic drug.

Furthermore, we performed a number of exploratory analyses in order to better dissect the role of these genes in psychopathological features. Firstly, we investigated the effects of the SNPs on clinical improvement measured by the PANSS subscales (i.e., positive, negative, and general subscales). Secondly, we investigated the relationships among the SNPs and (1) psychopathological severity at baseline, as measured by PANSS and its subscales; (2) age of onset; (3) family history of psychiatric disorders; and (4) suicide history.

All p values were two-tailed. In the main analyses (i.e., the case-control and pharmacogenetics analyses), the Bonferroni's correction was applied, taking into account the number of SNPs investigated in each sample (i.e., after preliminary analyses, 49 SNPs for the Korean sample and 21 SNPs for the Italian sample). Thus, the statistical significance was set to the p value of 0.001 in the Korean sample and of to the p value of 0.002 in the Italian sample. We did not apply any statistical correction to the exploratory analyses because they were performed only to better elucidate the role of the investigated genes to suggest further investigations. With these parameters ($p = 0.001$ and 0.002, respectively), we had a

sufficient power (0.80) to detect a small-medium effect size ($w = 0.19$ and 0.24, respectively) in both the Korean and Italian samples. Although the power of the samples in exam is clearly inadequate for detecting the very small effect typically attributed to the genetic risk variants for SCZ, the strong a priori selection of the genes and variants investigated should guarantee from false negative findings. Thus, the present study may help to better elucidate their role in SCZ, paving the way for further specific investigations.

Results

Sociodemographic Features

The sociodemographic features of the two samples are reported in Table 1. In both samples, patients and controls differed with respect to gender (Korean sample: $\chi^2 = 7.56$, df = 1, $p = 0.006$; Italian sample: $\chi^2 = 6.01$, df = 1, $p = 0.01$), and to age (Korean sample: $F = 45.5$, $p < 0.01$; Italian sample: $F = 1012.8$, $p < 0.01$). Clinical features investigated in exploratory

Table 1 Sociodemographic and clinical characteristics of the samples

Variable		Korean sample		Italian sample		
		Controls ($n = 326$)	Schizophrenia ($n = 176$)	Controls ($n = 194$)	Schizophrenia ($n = 83$*)	
					Schizophrenia baseline	Schizophrenia and response
Gender	Males	147 (45.1%)	102 (57.9%)	88 (45.4%)	44 (60.3%)	32 (62.7%)
	Females	179 (54.9%)	74 (42.0%)	106 (54.6%)	29 (39.7%)	19 (37.2%)
Age (years)		45.36 ± 13.09	37.19 ± 12.67	83.4 ± 7.1	42.3 ± 13.96	40.9 ± 14.21
PANSS total score	Baseline		94.46 ± 14.26		80.23 ± 23.93	85.69 ± 19.61
	Discharge		75.84 ± 8.85			64.00 ± 20.01
PANSS positive score	Baseline		24.74 ± 4.72		21.12 ± 7.97	23.39 ± 7.10
	Discharge		19.74 ± 4.04			16.83 ± 7.94
PANSS negative score	Baseline		21.89 ± 5.32		18.66 ± 7.82	19.37 ± 7.90
	Discharge		20.19 ± 4.20			15.65 ± 6.66
PANSS general score	Baseline		47.83 ± 8.24		40.45 ± 13.08	42.92 ± 10.91
	Discharge		35.90 ± 6.02			31.52 ± 8.47
Age at onset (years)	Estimated		28.76 ± 11.47		24.36 ± 9.09	24.58 ± 9.50
Duration of illness (years)			9.41 ± 10.79		15.64 ± 12.80	13.89 ± 12.85
Duration of admission (days)			37.5 ± 17.08		NA	10
Family history of psychiatric disorders	Yes		29 (16.5%)		28 (38.3%)	17 (33.3%)
	No		147 (83.5%)		25 (34.2%)	21 (41.2%)
	Missing				20 (27.4%)	13 (25.5%)
Suicide attempts	Yes		33 (18.7%)		17 (23.3%)	33 (64.7%)
	No		143 (81.2%)		38 (52.0%)	9 (17.6%)
	Missing				18 (24.6%)	9 (17.6%)
Antipsychotic drug	Risperidone		23 (13.1%)		19 (28.8%)	
	Olanzapine		108 (61.4%)		2 (3.0%)	
	Quetiapine		45 (25.6%)		2 (3.0%)	
	Clozapine				9 (13.6%)	
	Haloperidol				24 (36.4%)	
	Aripiprazole				5 (7.6%)	
	Others				12 (18.2%)	
Education (years)					12.41 ± 4.70	12.10 ± 4.86
Substance abuse	No				34 (46.6%)	24 (47.0%)
	Alcohol				3 (4.1%)	2 (3.9%)
	Illicit drugs				17 (23.4%)	15 (29.4%)
	Missing				19 (26.0%)	10 (19.6%)

*Some patients with incomplete data

analyses did not show any reciprocal association (data not shown). In case of positive findings, age and sex were added as covariate in the analysis.

Hardy-Weinberg Equilibrium and SNPs Frequencies

Korean Sample

In the Korean sample, all the SNPs were in HWE, except for rs1403737 within the RORA gene and rs7168443 within the ST8SIA2 gene (see Table 2). Further, for rs1673319 and rs17270745 within the RORA gene and for rs17522085 within the ST8SIA2 gene, a very small genetic variation was observed in the Korean sample (< 0.5% minor allele frequency). Therefore, these SNPs were excluded from further analyses. The SNPs included in the further analyses showed frequencies strictly comparable with the ones reported in the HapMap database (http://hapmap.ncbi.nlm.nih.gov/) for the reference populations (i.e., percentage difference < 2%).

Several SNPs investigated were in linkage disequilibrium (see supplementary Fig. 1).

Italian Sample

In the Italian sample, all the considered SNPs were in HWE, except for rs11780915, rs10108011, and rs2249098 within the PPP3CC gene, rs3759917 within the ST8SIA2 gene, and rs7603001 within the ZNF804A gene (see Table 2). Nonetheless, only rs3759917 showed allelic frequencies significantly different from reference populations (i.e., percentage difference > 2%), while for the other four SNPs the frequencies measured in the Italian sample were strictly similar to the ones reported in the HapMap database (http://hapmap.ncbi.nlm.nih.gov/) for the reference populations (i.e., percentage difference < 2%). Therefore, only rs3759917 was excluded from further analysis.

Several SNPs investigated were in linkage disequilibrium (see supplementary Figs. 1 and 2).

Case-Control Study

Korean Sample

In the case-control analysis, no association survived to the Bonferroni's correction. However, we found a nominal association between RORA rs10438338 and SCZ (genotypic analysis (g.a.), $p = 0.03$; allelic analysis (a.a.), $p = 0.07$).

In the haplotype analysis, the haplotype rs2282888-rs2237304-rs10272006-rs12673091 within the SP4 gene was associated with SCZ (global p value = 0.02; simulated $p = 0.02$).

Italian Sample

In the case-control analysis, PPP3CC rs11780915 (g.a. $p = 0.006$; a.a. $p = 0.001$) and rs2249098 (g.a. $p = 0.0004$; a.a., $p = 0.00006$) were associated with SCZ. The other associations found did not survive to the Bonferroni's correction. However, also PPP3CC rs10108011 was nominally associated with SCZ (g.a. p = 0.02; a.a., $p = 0.01$). Further, other nominal associations with SCZ were found for ZNF804A rs7603001 (g.a. $p = 0.07$; a.a. $p = 0.01$) and rs1344706 (g.a. $p = 0.02$; a.a. $p = 0.005$); SP4 rs12673091 (g.a. $p = 0.02$; a.a. $p = 0.06$); and ST8SIA2 rs4777989 (g.a. $p = 0.06$; a.a. $p = 0.01$).

In the haplotype analysis, the haplotype rs11780915-rs10108011-rs2249098 within the PPP3CC gene was associated with SCZ (global p value = 0.03; simulated $p = 0.03$). Sliding window analysis showed a major contribution for the haplotype rs10108011-rs2249098 (global p value = 0.002; simulated $p = 0.001$). Further, also haplotype rs7603001-rs1344706 within the ZNF804A gene was associated with SCZ (global $p = 0.02$; simulated $p = 0.01$).

Pharmacogenetic Study

Korean Sample

In the pharmacogenetics analysis, no association survived to the Bonferroni's correction. We found only nominal association with AR for rs1871858 (g.a. $p = 0.02$; a.a. $p = 0.006$), rs12900122 (g.a. $p = 0.06$; a.a. $p = 0.03$), rs17204440 (g.a. $p = 0.02$; a.a. $p = 0.02$), and rs9806453 (g.a. $p > 0.08$; a.a. $p = 0.03$) within the RORA gene, and for rs4777989 within the ST8SIA2 gene (g.a. $p = 0.09$; a.a. $p = 0.03$).

In the haplotype analysis, the haplotype rs1020729-rs1871858 within the RORA gene was associated with AR (global p value = 0.01; simulated $p = 0.01$).

Italian Sample

Concerning the pharmacogenetic analysis, also in the Italian sample, no association survived to the Bonferroni's correction. However, nominal associations with AR were found for rs12900122 within the RORA gene (g.a. $p = 0.003$; a.a. $p = 0.004$); rs2282888 (g.a. $p = 0.02$; a.a. $p = 0.002$), rs10272006 (g.a. $p = 0.02$; a.a. $p = 0.002$), and rs12673091 (g.a. $p > 0.08$; a.a. $p = 0.01$) within the SP4 gene; and rs4777989 within the ST8SIA2 gene (g.a. $p = 0.04$; a.a. $p > 0.08$).

In the haplotype analysis, no association was found with AR.

Table 2 Genetic variants investigated and results

Korean SNPs	Italian	Position	Location	HWE's *p* value		Results					
						Korean			Italian		
				Kor.	Ita.	C-C	Pgx	Bsl	C-C	Pgx	Bsl
PPP3CC											
	rs11780915	22305017	Intron		0.06				$\chi2 = 10.31$ $p = 0.006$ (g) *$\chi2 = 10.51$* *$p = 0.001$ (a)*		P.POS $p = 0.02$
rs10108011	rs10108011	22320806	Intron	1.0	0.05			P.TOT $p = 0.04$	$\chi2 = 8.32$ $p = 0.016$ (g) $\chi2 = 6.08$ $p = 0.014$ (a)		Onset $p = 0.05$ P.POS $p = 0.04$
rs7430		22398414	3' UTR	0.40				Suicid. Hx. $p = 0.008$			
rs2249098	rs2249098	22400098	Downstream	1.0	0.01				*$\chi2 = 15.55$* *$p = 0.0004$ (g)* *$\chi2 = 16.26$* *$p = 0.00006$* *(a)*		Onset $p = 0.01$
RORA											
rs1657792		60923429	Promoter	0.40			P.NEG $p = 0.04$ (g) $p = 0.02$ (a)	P.POS $p = 0.04$			
rs11630262	rs11630262	60924766	Promoter	1.0	0.99			P.POS $p = 0.01$			
rs9806453		60943845	Intron	0.33			P.TOT $p = 0.03$ (a) P.NEG $p = 0.06$ (g) $p = 0.01$ (a)	P.POS $p = 0.04$			
rs2553235		60969069	Intron	0.22			P.POS $p = 0.01$ (g) $p = 0.002$ (a)	P.POS $p = 0.0004$			
rs1871858		61047117	Intron	0.62			P.TOT $p = 0.02$ (g) $p = 0.006$ (a) P.NEG $p = 0.06$ (g) $p = 0.02$ (a) P.GEN $p = 0.07$ (g) $p = 0.02$ (a)	P.TOT $p = 0.04$ P.NEG $p = 0.02$ Onset $p = 0.008$			
rs12900122	rs12900122	61055411	Intron	0.30	0.53		P.TOT $p = 0.03$ (a) P.POS $p = 0.03$ (g) $p = 0.01$ (a)			P.TOT $p = 0.003$ (g) $p = 0.004$ (a) P.POS $p = 0.01$ (g) $p = 0.005$ (a) P.NEG $p = 0.001$ (g) $p = 0.01$ (a) P.GEN $p = 0.08$ (g) $p = 0.04$ (a)	
rs17204440		61066087	Intron	0.36			P.TOT $p = 0.02$ (g) $p = 0.02$ (a) P.GEN $p = 0.02$ (g) $p = 0.03$ (a)	P.GEN $p = 0.02$			
rs341382		61117831	Intron	0.92			P.NEG $p = 0.06$ (g) $p = 0.02$ (a)				
rs8041466		61155328	Intron	0.21			P.NEG	P.NEG			

Table 2 (continued)

Korean SNPs	Italian	Position	Location	HWE's p value		Results					
						Korean			Italian		
				Kor.	Ita.	C-C	Pgx	Bsl	C-C	Pgx	Bsl
							$p = 0.04$ (g)	$p = 0.04$ Suicid Hx $p = 0.01$			
rs12148149		61206551	Intron	0.40				Onset $p = 0.01$			
rs809736	rs809736	61329788	Intron	0.07	0.64					P.GEN $p = 0.04$ (g)	Fam Hx $p = 0.02$
SP4											
rs2282888	rs2282888	21476132	Intron	0.43	0.65			Onset $p = 0.01$		P.TOT $p = 0.02$ (g) $p = 0.002$ (a) P.POS $p = 0.03$ (g) $p = 0.002$ (a) P.GEN $p = 0.04$ (g) $p = 0.002$ (a)	
rs2237304		21499093	Intron	0.09			P.POS $p = 0.08$ (g) $p = 0.04$ (a)				
rs10272006	rs10272006	21520132	Intron	0.49	0.97			Onset $p = 0.01$		P.TOT $p = 0.02$ (g) $p = 0.002$ (a) P.POS $p = 0.03$ (g) $p = 0.002$ (a) P.GEN $p = 0.04$ (g) $p = 0.002$ (a)	
rs12673091	rs12673091	21538067	Intron	0.40	0.15				$\chi2 = 7.86$ $p = 0.02$ (g) $\chi2 = 3.46$ $p = 0.06$ (a)	P.TOT $p = 0.01$ (a) P.POS $p = 0.02$ (a) P.GEN $p = 0.005$ (a)	
rs9648275		21554464	Downstream	0.96			P.NEG $p = 0.04$ (g) $p = 0.05$ (a)				
ST8SIA2											
rs2305561		92987938	Coding exon	1.0				P.POS $p = 0.04$			
rs3784723	rs3784723	93005759	Intron	0.80	0.92						P.NEG $p = 0.04$
rs4777989	rs4777989	93006740	Intron	0.28	0.16		P.TOT $p = 0.03$ (a) P.NEG $p = 0.01$ (g) $p = 0.007$ (a)		$\chi2 = 5.48$ $p = 0.06$ (g) $\chi2 = 6.03$ $p = 0.01$ (a)	P.TOT $p = 0.04$ (g) P.POS $p = 0.04$ (g) $p = 0.05$ (a) P.NEG $p = 0.07$ (g) $p = 0.04$ (a)	
rs11853992	rs11853992	93012351	Downstream	0.61	0.88			Suicid. Hx. $p = 0.01$			P.NEG $p = 0.02$
rs17522085	rs17522085	93012557	Downstream	1.0	0.55	NA	NA	NA			P.NEG $p = 0.05$
ZNF804A											
rs7597593		185533580	Intron	0.80			P.NEG $p = 0.04$ (g)	P.GEN $p = 0.04$			
rs1987025		185647595	Intron	0.29			P.NEG $p = 0.03$ (g) $p = 0.03$ (a)				

Table 2 (continued)

Korean SNPs	Italian	Position	Location	HWE's *p* value		Results					
						Korean			Italian		
				Kor.	Ita.	C-C	Pgx	Bsl	C-C	Pgx	Bsl
rs7588907		185735599	Intron	0.48				P.NEG $p = 0.03$			
	rs7603001	185766816	Intron		7.0E-4				$\chi2 = 5.22$ $p = 0.07$ (g) $\chi2 = 6.27$ $p = 0.01$ (a)		
rs1344706	rs1344706	185778428	Intron	0.61	0.32				$\chi2 = 7.82$ $p = 0.02$ (g) $\chi2 = 8.00$ $p = 0.005$ (a)		Suicid. Hx. $p = 0.05$

(g) genotypic analysis, *(a)* allelic analysis, *Kor.* Korean, *Ita.* Italian, *HWE* Hardy-Weinberg Equilibrium, *C-C* case-controls, *Pgx* pharmacogenetics, *Bsl.* baseline, *NA* not available/not applicable, *P. TOT* PANSS total, *P. POS* PANSS positive, *P. NEG* PANSS negative, *P. GEN.* PANSS general, *Suicid. Hx* suicide history, *Fam. Hx.* familiar psychiatric history

[a] Data from www.snpper.chip.org. Only associations and trend of associations were reported in tables. In italics the associations which survived to the Bonferroni's correction

Exploratory Analyses

Exploratory Pharmacogenetic Analysis

In order to better elucidate the role of the SNPs investigated on AR, we investigated also their associations with the symptom clusters improvement measured by the PANSS subscales (i.e., positive, negative, and general subscales) (for details, see Table 2).

Korean Sample

Several SNPs within the RORA genes resulted nominally associated with improvement at the PANSS subscales, mainly with the negative subscale. More in detail, five SNPs (rs1657792, rs9806453, rs1871858, rs341382, and rs8041466) were associated with negative symptoms response, two SNPs (rs2553235 and rs12900122) with positive symptoms response, and two SNPs (rs1871858 and rs17204440) with general symptoms response. Within the SP4 gene, rs2237304 and rs9648275 were nominally associated with positive and negative symptom responses, respectively. Finally, ST8SIA2 rs4777989 and ZNF804A rs7597593 and rs1987025 were nominally associated with negative symptoms response.

In the haplotype analysis, two haplotypes within the RORA gene (i.e., rs1657792-rs8040067, global p value = 0.01; simulated $p = 0.01$, and rs1020729-rs1871858, global p value = 0.02; simulated $p = 0.02$) were associated with negative symptoms response. On the other hand, the haplotype rs3784723-rs3784722 within the ST8SIA2 gene was associated with general symptoms response.

Italian Sample

In the Italian sample, RORA rs12900122 was nominally associated with improvement at PANSS positive, negative, and general subscales. Within the same gene, rs809736 was nominally associated with general symptoms response. SP4 rs2282888, rs10272006, and rs12673091 and ST8SIA2 rs4777989 were nominally associated with both positive and general symptoms responses.

In the haplotype analysis, no association was found with symptom clusters AR.

Exploratory Analysis of Baseline Features

In order to obtain a deeper understanding of the effect of the genetic variants investigated, we performed further exploratory analyses on the baseline features of our samples. In order to avoid an excessive number of analyses, we limited to genotype analysis (for detail see Table 2).

Korean Sample

Within the PPP3CC gene, rs10108011 was associated with psychopathology severity at baseline and rs7430 with suicide history.

Within the RORA gene, rs1871858 was associated with psychopathology severity at baseline. Further, four SNPs near the gene promoter (i.e., rs1657792, rs11630262, rs9806453, and rs2553235) were associated with positive symptoms severity at baseline. On the other hand, rs1871858 and rs8041466 were associated with baseline negative symptoms. Finally, rs1871858 and rs12148149 were associated with SCZ

onset, rs8041466 with suicide history, and rs17204440 with general symptoms severity.

Concerning the SP4 gene, rs2282888 and rs10272006 were associated with SCZ onset.

Within the ST8SIA2 gene, rs2305561 was associated with positive symptoms severity at baseline and rs11853992 with suicide history.

Finally, ZNF804A rs7597593 was associated with general symptoms and rs7588907 with negative symptoms at baseline.

Italian Sample

Within the PPP3CC gene, two SNPs (rs2249098 and rs10108011) were associated with SCZ onset and two SNPs (rs10108011 and rs1178091) with positive symptoms severity at baseline.

Within the RORA gene, rs809736 was associated with psychiatric family history.

No association was found for SNPs within the SP4 gene.

Within the ST8SIA2 gene, three SNPs were nominally associated with negative symptoms severity at baseline (rs3784723, rs11853992, rs17522085).

Finally, ZNF804A rs1344706 was associated with suicide history.

Discussion

Case-Control Study

Concerning the PPP3CC gene, rs11780915 and rs2249098 were associated with SCZ in the Italian sample. Interestingly, we replicated previous finding about rs11780915, confirming the G allele as risk variant for SCZ (Sacchetti et al. 2013). Concerning rs2249098, although the association was not confirmed in the Korean sample and this variant has never been investigated in SCZ, it was associated with antidepressant response (Fabbri et al. 2014), suggesting a putative role for it in psychiatric disorders. Although the association did not survive to the Bonferroni's correction, rs10108011 was also nominally associated with SCZ in the Italian sample, with higher percentage of G allele in patients, thus partially confirming previous findings (Horiuchi et al. 2007; Kinoshita et al. 2005; Kyogoku et al. 2011). Interestingly, in the Korean sample, the G allele was also more present among the SCZ subjects (G allele: in controls 25.2%, in SCZ 29.3%), although it did not reach the statistical significance. Haplotypes analysis further supports the role of the PPP3CC gene in SCZ since the haplotype rs11780915 G - rs10108011 G - rs2249098 T was associated with SCZ as well (haplotype frequency 0.41, haplotype score 2.42).

Concerning the RORA gene, we found only a nominal association between rs10438338 and SCZ in the Korean sample in the genotypic analysis. No other association was found for this gene in both samples, suggesting that it may not have a major role in SCZ risk.

On the other hand, convergent nominal associations were found for SP4 gene variants, despite the fact that they did not survive to Bonferroni's correction. However, rs12673091 was nominally associated with SCZ in the Italian sample, as well as the haplotype rs2282888-rs2237304-rs10272006-rs12673091 in the Korean sample, thus supporting the relevance of the SP4 gene, and in particular of rs12673091, in SCZ. Our findings partially replicated the results by Zhou et al., which reported an association among this variant and both bipolar disorder (BD) and SCZ (Zhou et al. 2009), although in our samples the G allele resulted to be the risk variant. This discrepancy may be due to the different ethnicity of our samples compared to the one investigated by Zhou et al. (2009). Finally, two studies found SP4 gene variants associated with major depressive disorder (MDD) (Shi et al. 2011; Shyn et al. 2011), thus suggesting further investigations to better elucidate its role in psychiatric disorders. Indeed, the observed associations with different disorders may suggest that this gene may play a role in a wide range of psychiatric conditions.

For the ST8SIA2 gene, we found only a weak association between rs4777989 and SCZ in the Italian sample, which did not survive the Bonferroni's correction. Although to the best of our knowledge this variant has never been associated with SCZ, it is relatively close to a genetic region previously associated with both BD and SCZ (McAuley et al. 2012). On the other hand, we did not find any association with variants within the ST8SIA2 promoter, contrary to previous studies (Arai et al. 2006; Tao et al. 2007).

Finally, within the ZNF804A gene, two SNPs (rs7603001 and rs1344706) were nominally associated with SCZ in the Italian sample, while no association was found in the Korean sample. Although the association between rs7603001 was weak and is likely to be a false positive result (also taking into account that it was not in HWE), our result about rs1344706 replicated several previous findings about the association between the T allele and SCZ (e.g., O'Donovan et al. 2008), thus supporting its role in SCZ.

Pharmacogenetic Study

For both PPP3CC and ZNF804A genes, we failed to find any association between the SNPs investigated and AR in both samples. Although to the best of our knowledge the PPP3CC gene variants have never been associated with AR, some studies showed an association of ZNF804A genetic variants, particularly rs1344706, and AR (Mossner et al. 2012; Xiao et al. 2011; Zhang et al. 2012). We failed to replicate this

finding in our samples, maybe because of the relative small size of our samples.

Concerning the RORA gene, we found several nominal associations among its variants and AR. In particular, rs1871858, rs12900122, rs17204440, and rs9806453 were associated with AR in the Korean sample, as well as the haplotype rs1020729-rs1871858. Consistently, rs12900122 was associated with AR also in the Italian sample. Despite the fact that the RORA gene is considered one of the risk gene for autism (which is thought to partially share genetic background with SCZ (Murdoch and State 2013)) and it was associated with antidepressant response (Garriock et al. 2010; Hennings et al. 2015), to the best of our knowledge, it has never been investigated in the antipsychotic pharmacogenetics field. Thus, our preliminary results suggest that this gene, and particularly its variant, rs12900122, may modulate AR. Obviously, since our findings did not survive to the statistical correction, further investigations are needed to confirm RORA role in AR.

Similarly, ST8SIA2 rs4777989 was nominally associated with AR in both samples. In a previous study, this variant was associated with treatment tolerability in BD patients, which were also treated with antipsychotics (Fabbri et al. 2015). Thus, rs4777989 may modulate the effectiveness of antipsychotic treatments, although further studies are required to confirm this preliminary finding.

Finally, in the Italian sample, we found rs2282888, rs10272006, and rs12673091 within the SP4 gene associated with AR, although no association was found in the Korean sample. Since also this gene has not been investigated in the field of antipsychotic pharmacogenetics, further investigations are needed to confirm these preliminary findings.

Exploratory Analyses

Exploratory Pharmacogenetics Analyses

Consistent with the main pharmacogenetics analyses, we failed to find any association among PPP3CC variants and AR. Concerning the ZNF804A gene, we failed to replicate the association between rs1344706 and positive symptoms response (Mossner et al. 2012), although rs7597593 and rs1987025 showed weak associations with negative symptoms response in the Korean sample. Thus, further studies are needed to better dissect the role of this gene in AR.

On the other hand, we found some interesting results about the RORA gene. As a matter of fact, several variants within this gene were associated with negative symptoms response in at least one sample, as well as two haplotypes within the Korean sample. Furthermore, two other variants were associated with positive symptoms response (rs12900122 in both samples) and three with improvement at the PANSS general subscale, although these associations are weaker and less consistent across samples. Thus, our preliminary results further support a role for the RORA gene in AR, in particular for rs12900122. Previous finding about the RORA gene role in antidepressant response (Garriock et al. 2010; Hennings et al. 2015) may support our results, since negative and depressive symptoms are partially related (Schennach et al. 2015). However, further investigations are needed to better dissect the effect of the RORA gene on the AR of different symptom clusters.

Concerning SP4 gene variants, our exploratory analyses suggested that they might modulate the response of positive and general symptoms, rather than negative ones. In particular, in the Italian sample, the three SNPs nominally associated with AR in the primary analysis were associated with both positive and general symptoms improvement. In the Korean sample, rs2282888 was associated with improvement of positive symptoms, while rs9648275 was associated with improvement of negative symptoms. Consistent with our findings, previous preclinical studies showed that altered SP4 function leads to a high response to pro-psychotic agent, such as ketamine (Ji et al. 2013), thus supporting a role for this gene in the genesis of psychotic symptoms. Taken together, these results may suggest a role for this gene in positive symptoms response.

Finally, exploratory analyses on ST8SIA2 support a role for rs4777989 in AR, mainly on negative symptoms (with consistence of results across samples). Consistently, preclinical data showed that ST8SIA2 KO mice show a phenotype characterized by impaired working memory, deficits in prepulse inhibition, and anhedonic behavior, which are animal models of negative symptomatology (Krocher et al. 2015). However, an effect on other symptom clusters could not be ruled out since an association with positive symptoms improvement was found in the Italian sample for the same SNP, and haplotype analysis showed an association between haplotype rs3784723-rs3784722 and general symptoms improvement.

Exploratory Analysis of Baseline Features

In the Italian sample, PPP3CC rs11780915 and rs10108011 were associated with higher positive symptoms at baseline, while PPP3CC rs10108011 and rs2249098 were associated with the age of onset. In the Korean sample, rs10108011 was associated with global psychopathological severity at baseline, while rs7430 was associated with suicide history. These findings together may further support a role for PPP3CC in SCZ etiopathogenesis (Kyogoku et al. 2011), in particular for rs10108011. On the other hand, our finding about rs7430 and suicide history may be partially consistent with previous findings about PPP3CC and depression and antidepressant response (Fabbri et al. 2014; Kautzky et al.

2015), suggesting that this variant may be linked to depressive symptomatology.

Concerning RORA gene variants, exploratory analyses further support their role in modulation of SCZ symptoms. In particular, in the Korean sample, the variants within the gene promoter seem to modulate positive symptoms at baseline, thus suggesting a role for this gene area in psychotic symptoms pathogenesis. A possible effect on SCZ onset was also suggested by two associations found with rs1871858 and rs12148149. The importance of the rs1871858 variant was further supported by its association with both global psychopathological severity and negative symptoms at baseline. Also, rs8041466 seems to modulate negative symptoms and it was associated with suicide history as well. Other findings were less consistent and difficult to link from a neurobiological or clinical perspective. Obviously, because of the lack of previous investigations and the exploratory nature of these analyses, further studies are needed to confirm these preliminary findings.

Further, two SNPs within the SP4 gene were associated with SCZ onset in the Korean sample. Taking into account that alterations of SP4 transcription was found in first-episode SCZ patients (Fuste et al. 2013), we could speculate that this gene may be involved in the psychopathological onset of SCZ, although this hypothesis needs further investigation to be supported.

Concerning ST8SIA2 gene variants, in the Italian sample, four SNPs were associated with negative symptoms severity at baseline and one of them, rs11853992, was associated with suicide history in the Korean sample. Thus, the effects of this gene on negative symptomatology may deserve further investigations. Other findings were less consistent and difficult to justify both from a neurobiological and a clinical perspective.

Finally, an effect of the ZNF804A gene on negative symptoms was suggested by our exploratory analysis, although more specific investigations are clearly needed to confirm this association.

Conclusion

In conclusion, our results further support the role of the PPP3CC gene in SCZ, thus confirming previous findings about rs10108011 and rs11780915. Our exploratory analyses suggested that this gene may modulate positive symptoms severity and SCZ onset. On the other hand, RORA gene variants, in particular rs12900122, seem to modulate AR. Exploratory analyses suggested that RORA variants may modulate both positive and negative symptoms response. Of course, further investigations are needed to confirm these preliminary findings. Concerning the SP4 gene, we partially confirmed previous results about its association with SCZ, in particular for rs12673091 (Zhou et al. 2009). Furthermore, this gene seems to be involved also in AR, mainly concerning positive symptoms, at least in Caucasian population. Together with the associations found among SP4 variants and SCZ onset, these findings may suggest a role for this gene in the liability to develop psychotic symptoms, particularly taking into account also preclinical findings (Ji et al. 2013). For the ST8SIA2 gene, our results did not support a role in SCZ, while an effect of its variants on AR has been found, in particular for rs4777989. Exploratory analyses suggested that this variant may be implicated mainly in negative symptoms modulation. Finally, our results about the ZNF804A gene partially support its role in SCZ etiopathogenesis, in particular for rs1344706, while previous results about its effects on AR were not replicated. However, exploratory analyses pointed out a putative role for this gene in the modulation of negative symptomatology severity.

Since the investigated genes are involved in key molecular processes for brain functioning, the effects of their variants may be observed in different neuropsychiatric disorders in a cross-diagnostic perspective (Hettema 2016). On the other hand, we cannot rule out the alternative hypothesis that these genes play a relevant role only in SCZ etiopathogenesis. Finally, we should keep in mind that genetic variants associated with different neuropsychiatric disorders (i.e., with a cross-diagnostic effect) may cause impairments in compensatory systems/mechanisms, rather than in different "core" psychopathological processes, which are more likely illness-specific (Levine 2013). Thus, also our findings may point out to impairments in compensatory processes due to the SNPs in exam. Clearly, further studies are needed to better elucidate this issue.

In conclusion, our results partially support and detail previous findings about the investigated genes, although the limitations of the study should be carefully taken into consideration (mainly the small sample sizes and the different ethnicities of the two investigated samples). The exploratory analyses performed may help to better understand the effects of previously identified risk genes in SCZ pathophysiology, suggesting specific area for further, specific investigations. In this way, in the GWAS era where enormous samples are required, classical datasets with a deep phenotyping may complement GWAS findings, by providing new insights about the role of the genes identified and paving the way for further investigations.

Acknowledgements The authors thank all the participants in the present study.

Funding This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI12C0003).

Compliance with Ethical Standards The study protocol was approved by the institutional review board (approval number HC10TISI0031). The

study protocols were approved by the local Ethical Committees and they have been performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki.

Conflicts of Interest The authors declare that they have no conflict of interest.

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