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Genetic association of the Phosphoinositide-3 kinase in schizophrenia and bipolar disorder and interaction with a BDNF gene polymorphism

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Abstract

Phosphoinositide-3-kinase, class III (PIK3C3) is a member of the phosphoinositide-3-kinases family, involved in cell signaling, membrane trafficking, and neurodevelopment. Previous studies have indeed shown an association between *PIK3C3* gene variants and both bipolar disorder (BD) and schizophrenia (SZ). Brain-derived neurotrophic factor (BDNF) is a neurodevelopmental factor, which can regulate the PI3K signaling pathway. Associations have been reported between BDNF gene polymorphisms and affective and psychotic disorders. The aim of the present study was to replicate an association between *PIK3C3* and *BDNF* gene variants in SZ and BD and a putative epistasis between the two genes. Patients meeting the DSM-IV criteria of BD and SZ were included in this study (98 BD and 79 SZ) as well as 158 healthy controls. Blood DNA was extracted and genotyping was performed either by the polymerase chain reaction (PCR) technique followed by enzymatic digestion or by the high-resolution melt (HRM) method. Genotype and haplotype association was assessed with the UNPHASED statistical program.The results showed one nominal association with BD ($P < 0.02$) and two risk haplotypes in both SZ ($P < 0.001$) and BP ($P < 0.0005$), which survived multiple testing correction. A modest interaction between a BDNF variant and PI3KC3 polymorphism was observed ($P < 0.04$).These preliminary results confirm the genetic association of PI3K gene variants with both SZ and BD, and support the hypothesis that SZ and BD share a genetic background.

Introduction

Phosphoinositide-3-kinases (PI3K) are a family of enzymes, which catalyze the addition of a phosphate group to the D3 position of the inositol ring of inositol glycerophospholipids (Fruman et al. 1998). These proteins are involved in the regulation of many cellular processes such as proliferation, survival, and vesicle trafficking (Toker and Cantley 1997; Falasca and Maffucci 2009). Three classes have been described according to their substrate specificity and sequence homology (Vanhaesebroeck and Waterfield 1999). Class I is the most diversified with more than 10 catalytic (PIK3C) and regulatory (PIK3R) subtypes. Class II (PIK3C2) is less well understood but presents three catalytic isoforms, while class III (PIK3C3) has only one known subtype (Baker and Koretzky 2008). For several years, attention has been exclusively focused on class I but there is increasing interest in class III PIK3C, because of its involvement in neurodevelopment along with its role in autophagy (Baker and Koretzky 2008). Many neurodevelopmental disorders, such as schizophrenia (SZ) and autism, arise as a consequence of subtle developmental abnormality (Waite and Eickholt 2010) Moreover, evidence has supported the existence of a complex interplay between autophagy, cell proliferation, and cell death during neural development in mammals (Cecconi et al. 2007). In addition, *PIK3C3* is located on chromosome 18q12.3, within the region that maps closely to markers D18S450 and D18S487, linked to SZ (Williams et al. 1999). The implication of genetic factors in SZ and bipolar disorder (BD) is now well established (Craddock et al. 2006). Unsurprisingly,

associations between *PIK3C3* gene variants in SZ and BD have been reported (Stopkova et al. 2004; Duan et al. 2005; Saito et al. 2005). These studies suggested that PIK3C3 is a putative candidate gene for SZ, BP, and other neurodevelopmental diseases.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, also known for its role in neurodevelopment and cell survival (Huang and Reichardt 2001). A number of studies have indicated that BDNF is implicated in major depression, mood disorder, and SZ (Duman 2005; Karege et al. 2002; Lu and Martinowitch 2008). Several studies have subsequently reported some *BDNF* gene polymorphisms associated with mood disorder and psychosis, particularly the rs6265 (A196G), which replaces a valine by a methionine residue at position 66 of the protein (Neves–Pereira et al. 2002; De Luca et al. 2008; Vincze et al. 2008) although others failed to replicate this association (Tochigi et al. 2006; Watanabe et al. 2006). Lastly, basic studies have shown functional interactions between the neurotrophins—BDNF or neurotrophin-3 (NT-3)—and the phosphoinositol-3 kinase signaling pathway (Kaplan and Cooper 2001; Simpson et al. 2003; Reichardt 2006).

In this study, we aimed to replicate the association between the *PIK3C3* promoter variant, rs3813065, in both SZ and BP. This SNP is located in a crucial promoter area that could control the expression of this gene by POU-containing transcription factors (Stopkova et al. 2004). Additionally, we tested two single nucleotide polymorphisms (SNPs) and assessed their allelic and haplotype associations: rs8095411 and a dinucleotide repeat (CA) polymorphism also located upstream, *PIK3C3*. In a second time, we assessed the putative genetic interaction between *PIK3C3* and *BDNF* in both disorders. Finally, we evaluated the overlap hypothesis between SZ and BD by highlighting genetic variants in common for these diseases (Craddock et al. 2006). Given the central place occupied by PI3K and BDNF proteins in signaling networks and their crucial role in neurodevelopment and synaptic plasticity, we aim to determine whether these two disorders show similar patterns of genetic association.

Materials and Methods

Subjects

Patients meeting DSM-IV criteria for BD ($N = 98$) or SZ ($N = 79$) and controls ($N = 158$) were included after they had given informed consent and approval of ethics committee had been obtained (Table 1). Both BD and SZ patients were recruited from consecutive admissions to the psychiatric unit of the Geneva University Hospitals (Geneva, Switzerland). All patients were descended from at least two generations of Caucasians, and were interviewed by trained psychiatrists or psychologists using the French version of the Diagnostic Interview for Genetic Studies (DIGS) or the Mini International Neuropsychiatric Interview (MINI) (Nurnberger et al. 1994; Preisig et al. 1999). Almost all bipolar patients were diagnosed as BD-I, except for subjects identified as BP-II (98% of BP-I). Controls were recruited from blood donors in Geneva Hospitals (Geneva, Switzerland) and met the criteria of the DIGS questionnaire for their inclusion. The mean age (±SD) was 35 ± 10, 42 ± 11, and 44 ± 12 years, for SZ, BD, and Controls, respectively. The female composition was 40%, 50%, and 44%, for SZ, BD, and Controls, respectively.

Table 1. Demographic data.

	Female (%)	Male (%)	Mean age ± SD (yrs)	Range
SZ patient ($N = 79$)	40 (50.6)	39 (49.4)	35 ± 10	20–66
BD patient ($N = 98$)	50 (46.3)	58 (53.7)	42 ± 11	20–67
Controls ($N = 158$)	44 (27.8)	114 (72.2)	44 ± 12	17–73

Genotyping

DNA was extracted from peripheral blood leukocytes by using of the Nucleon BACC 2 kit (Amersham Biosciences, GE Healthcare, Glatbrugg, Switzerland). The -432C>T (rs3813065) was genotyped by restriction digestion with the enzyme *SwaI* as described by Stopkova et al. (2004). The dinucleotide repeat polymorphism was identified by the UCSC genome browser (March 2006). This microsatellite is located on chromosome 18, between 939, 492, 926–939, 492, 962 bp. This genetic variant was amplified by polymerase chain reaction (PCR) on a 96-well plate thermal cycler (Biometra, Goettingen, Germany).

The following primers were used: 5′-ACCTTTTCCTACTTCAATTCACA-3′ type forward and 5′-TCCTAGAGAAGAGGTATGATGATGG-3′ type reverse. PCR reaction was carried out with 100 ng of genomic DNA using Hot Star *Taq* DNA polymerase (Eurobio, Brunschwig, Basel, Switzerland) in a 25 mL reaction mix containing 1× buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7), 0.10 mM dNTPs, 0.03 mM $MgCl_2$, 0.02 mM of each primer, 1U *Taq* polymerase. Amplification conditions were as follows: 95°C for 5 min, 25 cycles of 92°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. PCR products were analyzed by electrophoresis on a 10% polyacrylamide gel at 250 V for 150 min and visualized with ethidium bromide. Allele $(CA)_{11}$ was 88 bp, allele $(CA)_{12}$ was 90 bp, allele $(CA)_{13}$ was 92 bp, allele $(CA)_{14}$ was 94 bp, allele $(CA)_{16}$ was 98 bp, allele $(CA)_{17}$ was 100 bp, and allele $(CA)_{18}$ was 102 bp.

The SNP rs8095411 was identified by the *Ensembl* data bank and explored by high-resolution melt (HRM) assay using a Rotor-Gene 6000 instrument (Corbett Life Science, Australia). Amplicon sequence was analyzed by the Poland melting software program to predict melting behavior (http://www.biophys.uni-duesseldorf.de/local/POLAND//poland.html).

The secondary structures were checked by DINAMelt (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-folding).

The following primers were used: 5′-GAGCCTGCAAAAACTCAACA-3′ type forward and 5′-AACCCAGCTGTCAGGGAATA-3′ type reverse. PCR reaction was carried out with 100 ng of genomic DNA using Kappa 2G Robust Hot Start Kit (Kappa Biosystem, Cape Town, SA) in a 20 μl reaction mix containing 0.2× buffer 0.2 mM dNTPs, 7.5 mM of each primer, 0.01 mM Hot Start polymerase, and 1.0 mM Syto9 green fluorescent intercalating dye (Invitrogen, Eugene, OR). Amplification conditions were as follow: 95°C for 3 min, 45 cycles of 95°C for 10 sec, 64°C for 30 sec, and 72°C for 10 sec. Methylation status was identified by HRM set from 70°C to 95°C, with the temperature rising by 0.2°C per second.

BDNF G196A polymorphism (rs6265) was genotyped by PCR followed by restriction enzyme digestion. A 133-bp segment was amplified by PCR on a 96-well plate thermal cycler (Biometra). The following primers were used: F 5′-GAGGCTTGACATCATTGGCT-3′ type forward and 5′-CGTGTACAAGTCTGCGTCCT-3′ type reverse. Target sequences were amplified in a 25-μl reaction mix containing 100 ng of genomic DNA, 1U *Taq* polymerase (Eurobio), 1.5 mM $MgCl_2$, 200 nM dNTP, and 10 pmol of each primer. Amplification conditions were as follows: 95°C for 5 min, 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec. PCR products were then digested overnight with 4 U of Eco72I (MBI Fermentas Inc., Glen Burne, MD). Then fragments were separated on a 10% polyacrylamide gel at 250 V and visualized with ethidium bromide. The A-allele undigested product size was 113 bp (A-allele) and the G-allele showed two fragments of 78 and 35 bp.

Statistics

SNPs with Hardy–Weinberg equilibrium below 0.001 in either cases or controls were excluded from analysis.

Haplotype analyses were performed using logistic regression. All rare haplotypes with frequencies below 1% in both patients and controls were excluded from analyses. Interaction test and omnibus tests, which assessed global differences in haplotype distribution between SZ and controls, BD and controls, and SZ + BD and controls, were initially conducted. If significant (below *P*-value of 0.05), we then used a sliding window procedure to extract the core haplotype associated with the diseases.

Since we compared genotypic and allelic distributions of four polymorphic markers between controls and three case populations (BP, SZ, and the sum of the two populations), a correction for multiple testing was required. Two approaches were applied to correct for multiple nonindependent comparisons. First, we used the highly conservative Bonferroni correction taking into account the nonindependence of tests. We effectively tested four independent polymorphisms, as linkage disequilibrium (LD) between polymorphisms within the same gene was not so strong. Two independent tests were considered for the different case populations as the sum of both of them would be closely related to the two case populations taken separately and because only significant results obtained in the whole population would be looked at in the two subpopulations. In addition, four tests were considered for haplotype analyses (dinucleotide repeat polymorphism—rs3813065; dinucleotide repeat polymorphism—rs8095411; rs3813065–rs8095411; and dinucleotide repeat polymorphism—rs3813065–rs8095411). Finally, an additional test was added for the interaction term. Therefore, for a Bonferroni correction on the *P*-values, we used $P = 0.05/(2 \times (4 + 4) + 1) = 0.003$ as a threshold of significance.

We applied the false discovery rate (FDR) to quantify uncertainty across the multiple hypotheses tested in the three single marker tests and the multiple haplotype tests. The FDR *q*-value was therefore calculated, which denotes the expected proportion of false negatives among multiple findings. Based on the single marker and haplotype association results, the *q*-value for each of these nonindependent tests was calculated using the step-up procedure (Benjamini and Hochberg 1995). The *q*-value calculated in this way has been shown to retain desirable properties for multiple related tests in genetic association studies and can be intuitively interpreted in terms of posterior error probability.

Statistical power to detect associations was estimated using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/purcell/gpc/). Thus, we determined that the SZ sample ($N = 79$) had 41% power to detect a risk allele with 20% frequency using an additive genotype model at alpha of 0.003. The BD sample ($N = 109$) had 52% power using the same criteria. The entire sample had 67% power.

Results

Single markers analysis

Three *PIK3C3 gene variants* (two SNPs: rs3813065 and rs8095411 and one microsatellite, a CA-repeat) and one *BDNF gene variant* (*rs6265*) were analyzed. Single marker association analysis detected a significant difference in genotype and allelic distributions of the *PIK3C3* -432C>T (rs3813065) between BD patients and controls ($P = 0.025$, $P = 0.028$, respectively) (Table 2). This difference was mainly accounted for by lower frequencies of CT genotype and T-allele in BD than in controls. However, this association did not hold after Bonferroni correction for multiple testing. No other association was significant either for BDNF or PI3KC3 variants (Table 2). For the microsatellite variant, genotyping analysis revealed length polymorphisms in this $(CA)_n$ repeat, with n

Table 2. Allele and genotype distribution in SZ, BD, and control subjects for three polymorphisms of BDNF and PI3KC3 genes.

		Population						Statistics (*P*-value)		
		Controls		BD patient		SZ patient				
Variant	Genotype	*n*	Freq.	*n*	Freq.	*n*	Freq.	CT vs. BD	CT vs. SZ	CT vs. (BP + SZ)
BDNF	AA	9	0.057	3	0.028	4	0.051	0.47	0.75	0.748
rs6264	AG	56	0.354	42	0.385	32	0.405			
	GG	93	0.589	64	0.587	43	0.544			
	G	74	0.234	48	0.22	40	0.253	0.71	0.65	0.997
	A	242	0.766	170	0.779	118	0.747			
PIK3C3	CC	145	0.924	107	0.982	69	0.873	**0.025**	0.22	0.221
rs3813065	CT	12	0.076	2	0.018	10	0.127			
	TT	0	0	0	0	0	0			
	C	302	0.962	216	0.991	148	0.937	**0.028**	0.24	0.653
	T	12	0.038	2	0.009	10	0.063			
PIK3C3	AA	112	0.762	86	0.789	57	0.722	0.607	0.51	0.507
rs8095411	AG	35	0.238	23	0.211	22	0.279			
	GG	0	0	0	0	0	0			
	A	259	0.881	195	0.895	136	0.861	0.6316	0.54	0.98
	G	35	0.119	23	0.106	22	0.139			

Freq = frequency; CT = controls; BD = bipolar disorder; SZ = schizophrenia.
P-values that held after Bonferonni correction are in bold face.

ranging from 11 to 18. The most frequent alleles were $(CA)_{13}$ (56.4%), $(CA)_{16}$ (18.8%), $(CA)_{18}$ (14.9%), $(CA)_{12}$ (3.2%), $(CA)_{17}$ (3.2%), $(CA)_{14}$ (2.1%), and $(CA)_{11}$ (1.4%) (Information not displayed), but no association was significant either for BD or SZ.

Haplotype analysis

Omnibus tests comprising the three polymorphic markers, that is microsatellite, rs3813065, and rs8095411, from the 5′ to the 3′ end of the *PIK3C3* gene, showed significant differences in the overall haplotype distribution between controls and SZ (omnibus test: $P = 0.030$, $X^2 = 13.96$, df = 6), controls and BP (omnibus test: $P = 0.017$, $X^2 = 17.02$, df = 7), and controls and all patients (SZ + BP) (omnibus test: $P = 0.016$, $X^2 = 18.84$, df = 8). One three-marker haplotype, $(CA)_{13}$-C-G, mainly accounts for these differences. This haplotype showed higher frequencies in SZ ($P = 0.021$) and in BP ($P = 0.0075$) patients than in controls (Table 3). Interestingly, when all patients were pooled together, this risk haplotype remained significant ($P = 0.0016$) and held after Bonferroni corrections. To strengthen the power of our analysis and to highlight at-risk haplotypes, the analysis was then repeated with haplotypes formed by two-marker combinations excluding rs3813065. Omnibus tests showed more significant *P*-values in combined SZ and BP patients compared to controls (omnibus test: $P = 0.0032$, $X^2 = 23.11$, df = 8). SZ only and BP only versus controls showed the same tendency (omnibus tests: $P = 0.0084$, $X^2 = 17.25$, df = 6 and $P = 0.017$, $X^2 = 17.09$, df = 7, respectively). Moreover, even after corrections for multiple testing, one main haplotype $(CA)_{13}$-G was more frequent in the pooled population of SZ and BD patients than in controls ($P = 5.81 \times 10^{-4}$). This risk haplotype was also statistically significant in SZ patients ($P = 0.0011$) and showed a trend of association in BD patients ($P = 0.022$).

As multiple positive findings were expected and found, the FDR *q*-value was calculated to quantify the joint probability of multiple findings reflecting true associations as opposed to false positives, taking into account all comparisons performed to test the three hypotheses. The three top associations of the multiple comparisons (Table 2) were attributed a *q*-value of 0.09 or less. While, after multiple comparison corrections, it was not possible to reject all of the null hypotheses at a conventional level of statistical significance, all three of them were very unlikely to represent false positives.

Interaction analysis between PIK3C3 and BDNF variants

After the omnibus test, one interaction remained statistically significant between rs6265 (*BDNF*) and rs8095411 (*PIK3C3*) in SZ compared to controls (omnibus test: $P = 0.04637$, $X^2 = 3.968$, df = 1). This result is mainly supported by genotype interaction rs6265/ rs8095411 A/G-A/G (16.46% vs. 6.8%, $P = 0.028$). This interaction did not survive the Bonferroni test for multiple corrections.

Discussion

The aim of this case-control study was to assess the potential role of *PIK3C3* genetic variants in SZ and BD patients and its

Table 3. Haplotype analysis of the PIK3C3 gene in bipolar disorder (BD) and schizophrenia (SZ).

			Frequency				Statistics (*P*-value)		
Gene variant			CT	BD	SZ	BD + SZ	CT vs BD	CT vs SZ	CT vs (BD + SZ)
CA-repeat	rs3813065	rs8095411							
(CA)13	C	G	0.015	0.072	0.072	0.075	**0.007**	0.02	**0.001**
(CA)13		G	0.019	0.072	0.112	0.094	0.02	**0.001**	**0.0005**

P-values that held after Bonferonni correction are in bold face.; CT = controls; BD = bipolar disorder; SZ = schizophrenia.

possible interaction with a BDNF polymorphism. The analysis of the single marker yields a difference, albeit modest, in allele distribution of the rs3813065 (-432C>T) with respect to BD, but not SZ. This result replicates the previous study from Stopkova et al. (2004). Moreover, the minor allele distribution is similar to that described in previous publications, which reported that it was very rare in USA and Czech populations while homozygote was missing in Jewish populations in Israel (Stopkova et al. 2004). It is highly probable that these authors did not observe any change in allele distribution either in BD or SZ in USA populations compared to controls. The difference was observed only with Czech and Jewish populations (Stopkova et al. 2004), and later in African–American populations with SZ (Saito et al. 2005). This suggests that ethnic factors are at play within this promoter variant.

Interestingly, our study has observed multimarker haplotypes at risk for BD and SZ. In both diagnostic categories, the differences from controls were highly significant with a mean odds ratio (OR) exceeding a value of 2.5 in each individual diagnostic category and 3.0 in both diagnostic categories combined. Importantly, *P*-values remained significant after Bonferroni correction. Consistent with the reports above (Stopkova et al. 2004; Saito et al. 2005), this result supports the hypothesis that PI3KC3 gene variants are implicated in the etiology of SZ and BD. This observation is relevant as many neurobehavioral disorders arise as a consequence of subtle developmental abnormalities. The genetic alteration of an important neurobiological factor such as PI3KC could contribute to these disorders. Previous studies have shown that the neurobiology of inositol and related lipid kinases contributes to the pathophysiology of disorders such as SZ and autism (Waite and Eickholt 2010). Importantly, the fact that both BD and SZ diagnostics were similarly affected is in favor of the hypothesis of a shared genetic background in these diseases. Molecular genetics has recently challenged the strict dichotomy between BD and SZ, and a number of important studies have reported alterations in genes or gene products shared by these two disorders (Craddock et al. 2006; Shao and Vawter 2008).

Our study also evaluated a putative interaction between *PIK3C3* and a BDNF gene variant (*G196A)* in the two patient groups. The interaction between these two neurodevelopmental factors has been demonstrated in physiologic studies (Reichardt 2006). BDNF was reported to activate PI3KC and one of the BDNF–PI3K–AKT signaling pathways plays a pivotal role in the long-term maintenance of synaptic plasticity through translation and transport proteins (Sun et al. 2010). In contrast to previous reports, our study did not replicate the association of BDNF variant either in BD or in SZ. This is probably due to the small size of our population. In BD, although the allele distribution was nearly the same as in the previous study (Vincze et al. 2008), the latter emerged as highly significant, because of the greater number of patients, while the present study felt short statistically (Vincze et al. 2008). Considering the putative functional role of these proteins and their cell signaling interaction, we tested for a potential interactive effect of their polymorphisms in these disorders. A significant association, albeit modest, was observed in this epistasis evaluation.

In conclusion, this study must be considered as a preliminary investigation and results should be viewed with caution considering the errors associated with case-control studies of gene candidates. However, given the central place occupied by PI3KC protein in signaling networks and its crucial role in neurodevelopment, the study deserves to be replicated with larger samples. The possibility that gene variations are shared between these two major psychoses, and the putative epistasis with other important factors, such as BDNF, worth thorough investigation.

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