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Association analysis of polymorphisms of the NTRK2 and BDNF genes with bipolar affective disorder in a Polish sample

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Summary

Aims. Neurodevelopmental abnormality has been implicated in the pathogenesis of bipolar disorder. The neurotrophin BDNF regulates not only cell survival, proliferation and synaptic growth in the developing CNS, but also plays a crucial role in activity–dependent neuroplasticity. Substantial evidence has pointed to the role of the BDNF in the pathophysiology of mood disorders and in the mechanism of action of therapeutic agents. Consequently, BDNF and its receptor, encoded by the NTRK2 gene, constitute good candidates for molecular genetic studies in bipolar affective disorder (BPD).

Methods. In the present study we selected four single nucleotide polymorphisms (SNPs) of the BDNF and three SNPs of the NTRK2 genes. The case–controlled analyses were performed on patients with bipolar disorders (n=455, control n=589) and in the subgroups of patients, formed by using the following criteria: the clinical type of BPD (BPD type I & II), gender, age of onset, family history of (BPD) and a history of suicidal attempts.

Results. The haplotype GC (rs988748/rs203024) of the BDNF gene was significantly more frequent in patients with BPD then in controls (p=0.006). Also haplotype CA (rs 1187326/rs1187327) of the NTRK2 gene was significantly more frequent in patients than in the control group (p=0.046). Case-control analysis of single markers after adjusting the significance level for multiple tests did not show any significant differences.

Conclusions. These data suggest that polymorphisms of BDNF and NTRK2 genes may be involved in the aetiology of bipolar disorder in the Polish population.

bipolar affective disorder / neurodevelopmental hypothesis / neurotrophins / brain-derived neurotrophin factor (BDNF) / Neurotrophic Tyrosine Receptor Kinase / type 2 (NTRK2).

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Abbreviations: A - Adenine, BPD - bipolar disorder, BPDI - bipolar disorder type I, BPDII - bipolar disorder type II, BDNF - brain-derived neurotrophic factor, DNA – deoxyribonucleic acid, DSM-IV – Diagnostic and Statistical Manual, 4th ed., EDTA – ethylenediaminetetraacetic acid, G - Guanine, ICD-10 – International Classification of Disease, 10th ed., Met - Methionine, NTRK2 - Neurotrophic Tyrosine Receptor Kinase, type 2, PCR – polymerase chain reaction, RFLP – restriction fragment length polymorphism, Val - Valine

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INTRODUCTION

Bipolar disorder (BP) is characterised by profound mood symptoms that include episodes of mania, hypomania and depression. Moreover, the data from family and twin studies strongly demonstrate the contribution of genetic factors in the susceptibility to BP [1]. BP is a common disorder caused by the interaction of multiple genetic and environmental factors, but its aetiology is difficult to determine.

In 1987 Weinberger and Murray & Lewis independently proposed the neurodevelopmental hypothesis of schizophrenia which is an attempt to connect dysfunction in brain development with disturbances in many neurotransmitter systems, dysfunction of synaptic plasticity

and cognitive distortion [2, 3]. This hypothesis has universal meaning and can be extrapolated to all psychiatric disorders. Initial data supporting this theory in bipolar patients comes from a paper of Harrison et al. who identified a cellular pathology of neurodevelopment in postmortem brain tissue [4]. Recent studies using in vivo magnetic resonance imaging (MRI) in patients with BPD found evidence of early neurodevelopmental abnormalities in the anterior cingulate cortex [5]. According to this hypothesis, environmental factors such as maternal diabetes, stress, malnutrition, hypoxia, infections etc. present in the prenatal and early postnatal periods can cause disturbances in CNS development as well as influence expression of numerous genes potentially important in the pathogenesis of psychiatric disorders. Among the genetics factors, genes encoding for neurotrophins have also been proposed as candidate genes in BP [6, 7, 8, 9, 10]. During development, neurotrophic factors regulate proliferation, differentiation and migration of neurones. In the mature nervous system, neurotrophins influence neuronal survival, dendritic growth and synaptic plasticity [11]. Disturbances in the neurotrophin expression or function may cause structural abnormalities and deficiency in different neurotransmitters systems. In some expression studies, disturbances in Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), neurotrophin 3 (NT3) and their receptors were found in the different brain structures [12, 13, 14]. Numerous pieces of evidence suggest that reduced neurotrophic factor signalling in the adult brain is implicated in the pathophysiology of depressive episodes in bipolar disorders [15]. As a consequence of stressor activity a decrease in expression of neurotrophic factors in limbic structures is being observed. This effect can be reversed or blocked by antidepressant treatment. It has also been hypothesised that mania may be caused by the hyperactivity of the central BDNF function [16]. Thus, a kind of compensatory process may be occurring in the brain tissue. In depression, the amount of neurotrophins is also reduced. This decrease may lead, as a feedback mechanism, to an increased expression of neurotrophins and cause a shift into the manic phase. Those data make genes mentioned above attractive candidates for association studies in bipolar disorder. In this study we have focused on two functionally connected genes: BDNF and NTRK2 (encoding receptor Tyrosine Kinase B for BDNF). The NTRK2 gene is a promising candidate gene for BPD because of the BDNF/TrkB functional signalling pathway. BDNF acts via the tyrosine kinase (TrkB) receptor and affects transcription via mitogen-activated protein kinase (MAPK), phosphatydylinositol 3-kinase (PI3-K) and phospholipase C (PLC) α signal transduction pathway [17]. In animal models, mice with altered or knocked-out gene for TrkB are used to eliminate the BDNF function [18]. Recent studies have shown that knockdown of TrkB gene caused a decrease in embryonic precursor cell proliferation and disturbances in the postnatal localisation of cortical neurones [19]. Furthermore, synaptic formation was inhibited and synaptic plasticity was disturbed [20]. Stress, seizure, exercise, antidepressants and antipsychotic agents change in vivo expression of TrkB, which further supports the hypothesis that TrkB may be responsible for the predisposition to psychiatric disorders [20, 21].

Selected polymorphisms of the BDNF and NTRK genes have been frequently analysed in psychiatric disorders (schizophrenia [22, 23, 24], affective disorders [25, 26, 27, 28, 29, 30], addiction [31], in responses to lithium treatment [32], migraine [33], ADHD [34], eating disorders [35], and Alzheimer's disease [36, 37]). Only the rs6265 polymorphism of the BDNF gene was shown to be functional, were the substitution of adenine (A) for guanine (G) results in methionine (Met) substitution for valine (Val) at codon 66 (Val66Met). The other selected SNPs are located in introns and thus may influence splicing or gene expression. In this paper, we present a case-control study and a haplotype analysis for four single nucleotide polymorphisms (SNPs) of the BDNF gene (rs2030324, rs988748, rs6265, rs2203877) and three SNPs of the NTRK2 gene (rs1187326, rs2289656, rs1187327) in a sample of the Polish population. We hypothesised that variants of BDNF and NTRK genes may show association with BP or subtype of BP in particular with early onset of BP.

SUBJECTS AND METHODS

The study included 455 Polish unrelated individuals with bipolar affective disorder (BPD).

Patients were divided into subgroups based on clinical features: group with bipolar I affective disorder (BPDI n=369) and with bipolar II affective disorder (BPDII n=86). The analysis was performed separately on patients with the disease onset before and after 18 years of age. Family history, in the first and second degree relatives, of bipolar spectrum disorders (including bipolar disorder, schizoaffective disorder and/or major depression) was established in 24% of patients with BPDI and in 28% of patients with BPDII. History of suicide attempts was found in 21% of patients with BPDI and in 28% of patients with BPDII. Consensus diagnosis by two experienced psychiatrists, using DSM-IV and ICD-10 classification was made for each patient using the Structured Clinical Interview for DSM-IV Axis I disorders (SCID) [38]. The group was ethnically homogenous. Recruited patients were treated in the Department of Psychiatry, University of Medical Sciences in Poznań, in the Department of Psychiatry, University School of Medicine in Bydgoszcz and in the Psychiatric Hospital in Kościan.

The control group consisted of 589 subjects who were recruited among the blood donors, hospital staff and students of Poznań University of Medical Sciences. They were not psychiatrically screened. All patients gave written informed consent. The project was accepted by the local ethics committee. Clinical and demographic characteristics of the sample were presented in Tab. 1.

Table 1. Clinical and Demographic Characteristics of the Sample

Study group	Total BPD N=455		BPI N=369		BPDII N=86		Control N=589	
	N	A	N	A	N	A	N	A
Males	188	43.9±14.6	157	43.3±14.8	31	45.3±14.1	234	40.5±12.2
Females	267	45.9±12.6	212	46.3±14.0	55	46.2±12.7	355	40.1±11.5
Average of onset	455	30±11.4	369	29±11.0	86	34±12.0		
Family history	113 (25.0)		89 (24.0)		24 (28.0)			
Suicidal attempts	102 (22.0)		78 (21.0)		24 (28.0)			
Age of onset 18≤ years	54 (12.0)							

N - number of subjects (% of total subgroups of patients in brackets), A - mean age; value are means ± SD

Genotyping

DNA was extracted from 10 ml of EDTA anti-coagulated whole blood using the salting-out method [39]. Fragments of interest in the BDNF and NTRK2 genes were amplified by PCR with primer pairs presented in Tab. 2. For genotyping, a general protocol for all polymorphisms was used. Briefly, 250 ng of genomic DNA were amplified in 15 μl of a solution containing: 1xPCR buffer, 1.5 μM MgCl2, 0.45 μM primers, 170 μM dNTP, and 0.5 U Taq polymerase. The thermal profile was as follows: amplification at 95oC for 2 min., followed by thirty cycles consisting of denaturation at 95oC for 30s, annealing at the appropriate primer pair annealing temperature for 30s (Tab. 2) and extension at 72oC for 30s, with the final extension step of 5 min at 72oC. PCR product was digested overnight with the corresponding restriction endonucleases. Digestion products were then separated on 2.5% agarose gel. Size of DNA fragments were presented in Tab. 2 (*next page*).The genotyping was performed without knowledge of the subject's clinical status. The uncut PCR products for all SNPs were digested twice to confirm the results. The control of RFLP analysis was also performed (10% of the randomly chosen samples from both groups).

Statistical analysis

The Pearson's chi-square ($\chi 2$) test and Fisher's exact test were applied to test differences in the genotypic and allelic (respectively) distribution between groups of bipolar patients and controls.

Table 2. Primer design and PCR

Genes	SNP rs #	SNP's positions *	Annealing Tm °c	PCR Product bp	Restriction enzymes	Restriction products bp	Primer sequences	Source
BDNF	rs2203877 (intronic)	ch11: 27627486	63	223	HinfI	C: 127+78 T: 127+63	F: 5'CAG AGG AGC CAG CCC GGT GCG 3' R: 5' CTC CTG CAC CAA GCC CCA TTC 3'	Kunugi, 2004
	rs6265 (exonic)	ch11: 27636492	60	197	Eco72I (PmaCI)	G: 73+124 A: 197	F: 5`ACT CTG GAG AGC GTG AAT GG 3` R: 5` AGA AGA GGA GGC TCC AAA GG 3`	*
	rs988748 (intronic)	ch11: 27681321	64	178	Alw26I	G: 178 C: 148+30	F: 5' TTG GAG TAG GGT TCC TCC AGT 3' R: 5' AGA GGG CAT GAA GCT GGA TA 3'	Watanabe, 2006
	rs2030324 (intronic)	ch11: 27683491	64	348	TaiI	T: 348 C: 268+79	F: 5' TTG CAC ATC CTG CTC AAG TC 3' R: 5' TTG CTA GGA GAA AAG CCA TGA 3'	Vepsalainen, 005
NTRK2	rs1187326 (intronic)	ch9: 86475735	64	211	Eco 81I	C: 211 T: 86+125	F: 5' CCG AGA TTG GAG CCT AAC AG 3' R: 5' AAG CCA GTG AAA CAG CAG GT 3'	Vepsalainen, 2005
	rs1187327 (intronic)	ch9: 86478360	58	341	Eco47I (AvaII)	A: 341 G: 245+96	F: 5' GAT GTT GAG CAG GCG TGA TA 3' R: 5' GCA ACA CAC AAC TTG CTG AAA 3'	*
	rs2289656 (intronic)	ch9: 86753382	58	309	SsiI (AciI)	T: 309 C: 120+189	F: 5' CTG GCA TAT GCT GGT GTC AT 3' R: 5' CTG AAG GAT GCC AGT GAC AA 3'	*

*Chromosomal positions: Map to Genome Build 36.3 (http://www.ncbi.nlm.nih.gov/)
Primer were designed using „Primer3" software: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
*Primers previously not published were designed by dr Maria Skibińska.

Calculations were performed using STATISTICA v7.1 software. For multiple tests Bonferroni correction was applied using the formula: $\alpha` = \alpha/k$ where $\alpha`$ is the corrected significance level for multiple comparisons, α is the significance level (0.05) and k is the number of independent significance tests. Corrected significance level for the BDNF gene polymorphisms (for k=16 groups and k x (k-1)/2=120 comparisons) was 0.0004. The corrected significance level for multiple tests of the NTRK2 gene polymorphisms (k=12 groups and 66 comparisons) was 0.0007. GraphPad software was used (http://www.graphpad.com) to calculate odds ratios (OR) for the alleles. Power analysis was performed using an excel file power calculation prepared by Ph D José Osorio y Fortéa from Institut Pasteur Immunophysiologie et Parasitisme Intracellulaire. In this manner power analysis based on minimal detectable difference (MDD) between P1 and P2, corresponding to the percentage of the studied allele within the control and the case samples, respectively [40]. The "Utility Programs For Analysis Of Genetic Linkage" (Copyright © 1988 J. Ott) was used to test the Hardy-Weinberg equilibrium with p value <0.05. A two-tailed type I error rate of 5% was chosen for analysis. For linkage disequilibrium analysis of the analysed polymorphisms, the free on-line software case HaploView v 4.0 was used. (http://www.broat.mit.edu/mpg/haploview/download.php)

RESULTS

There were no significant deviations from the Hardy-Weinberg equilibrium in the group of patients with bipolar disorder and in the control group (rs2030324 for controls p=0.575, for patients p=0.943; rs988748 for controls p=0.237, for patients 0.650; rs6265 for controls p=0.123, for patients p=0.590; rs2203877 for controls p=0.093, for patients p=0.421; rs1187326 for controls p=0.341, for patents p=0.382; rs2289656 for controls p=0.546, for patients p=0.686; rs1187327 for controls p=0.950, for patients p=0.954)

The genotype distribution and allele frequencies of four selected SNPs of the BDNF gene in patients with BPD, BPDI, and BPDII were shown in Tab. 3 (*next page*); there were no significant differences in the genotypes and alleles frequencies in comparison to the control group. Also, no significant differences in genotypes and allele frequencies were found in the subgroups divided by gender.

The allele and genotypes frequencies in female and male groups of BPD, BPDI, BPDII groups were shown in Tab. 4 (*next page*). Comparing genotype and allele distribution according to the age of onset (≤18 or >18 years), we found that Met/Met genotype of the BDNF gene was more frequent in the subgroup of BPDI patients with an early age of onset (≤18 years) than in the controls (13.3% v. 2.6%); however, the difference did not reach sig-

Table 3. Genotype and allele frequencies of polymorphisms of the BDNF gene

	n	genotype			p	allele		p
rs2030324		C/C	C/T	T/T		C	T	
control	568	161 (28.4)	289 (50.9)	118 (20.7)		611 (53.8)	525 (46.2)	
Total BPD	403	121 (30.0)	199 (49.4)	83 (20.6)	0.884	441 (54.7)	365 (44.3)	0.711
Bipolar I	320	93 (29.1)	163 (50.9)	64 (20.0)	0.952	349 (54.5)	291 (45.5)	0.766
Bipolar II	83	28 (33.7)	36 (43.4)	19 (22.9)	0.428	92 (55.4)	74 (44.6)	0.739
	n	genotype			p	allele		p
rs988748		C/C	C/G	G/G		C	G	
control	583	355 (60.9)	206 (35.3)	22 (3.8)		916 (78.6)	250 (21.4)	
Total BPD	452	282 (62.4)	148(32.7)	22 (4.9)	0.519	712 (78.8)	192 (21.2)	0.954
Bipolar I	362	227 (61.9)	123 (33.5)	12 (4.6)	0.721	577 (78.6)	157 (21.4)	1.000
Bipolar II	85	55 (64.7)	25 (29.4)	5 (5.9)	0.418	135 (79.4)	35 (20.6)	0.841
	n	genotype			p	allele		p
rs6265		G/G	G/A	A/A		G (Val)	A (Met)	
control	585	380 (65.0)	190 (32.5)	15 (2.6)		950 (81.2)	220 (18.8)	
Total BPD	379	260 (68.6)	106 (28.0)	13 (3.4)	0.275	626(82.6)	132(17.4)	0.469
Bipolar I	321	219 (68.2)	91 (28.4)	11 (3.4)	0.365	529 (82.4)	113 (17.6)	0.568
Bipolar II	58	41 (70.7)	15 (25.9)	2 (3.5)	0.563	97 (83.6)	19 (16.4)	0.616
	n	genotype			p	allele		p
rs2203877		C/C	C/T	T/T		C	T	
control	501	444 (88.6)	53 (10.6)	4 (0.8)		491 (88.9)	61 (11.1)	
Total BPD	412	370 (89.8)	40 (9.7)	2 (0.5)	0.763	780 (94.7)	44 (5.3)	0.544
Bipolar I	326	290 (89.0)	34 (10.4)	2 (0.6)	0.951	614 (94.2)	38 (5.8)	0.915
Bipolar II	86	80 (93.0)	6 (7.0)	0 (0.0)	0.407	166 (96.5)	6 (3.5)	0.213

Numbers in brackets indicate percentages

Table 4. P value for comparison of genotype and allele distribution of each BDNF polymorphism according to gender

	n	male		n	female	
		genotype	allele		genotype	allele
rs2030324						
		C/C C/T T/T	C&T		C/C C/T T/T	C&T
control	215			352		
Total BPD	166	0.651	0.378	237	0.388	0.234
Bipolar I	136	0.381	0.243	184	0.358	0.220
Bipolar II	30	0.233	0.103	53	0.891	0.834
rs988748						
		C/C C/G G/G	C&G		C/C C/G G/G	C&G
control	229			353		
Total BPD	186	0.711	0.452	266	0.117	0.884
Bipolar I	156	0.695	0.540	211	0.154	0.542
Bipolar II	30	0.612	0.626	55	0.359	0.798
rs6265						
		G/G G/A A/A	G&A		G/G G/A A/A	G&A
control	231			354		
Total BPD	168	0.602	0.530	211	0.363	0.682
Bipolar I	147	0.834	0.710	174	0.277	0.602
Bipolar II	21	0.260	0.422	37	0.998	1.00

(*table continued on next page*)

rs2203877						
		C/C C/T T/T	C&T		C/C C/T T/T	C&T
control	178			322		
Total BPD	178	0.438	0.448	234	0.876	1.00
Bipolar I	147	0.556	0.565	179	0.852	0.700
Bipolar II	31	0.484	1.00	55	0.627	0.528

nificance (p=0.034 for genotypes; p=0.486 for alleles; data not shown).

The analysis performed according to the family history of bipolar disorders and suicidal attempts did not show any significant differences in the genotype and allele frequencies for any of the analysed SNPs of the BDNF gene (data not shown).

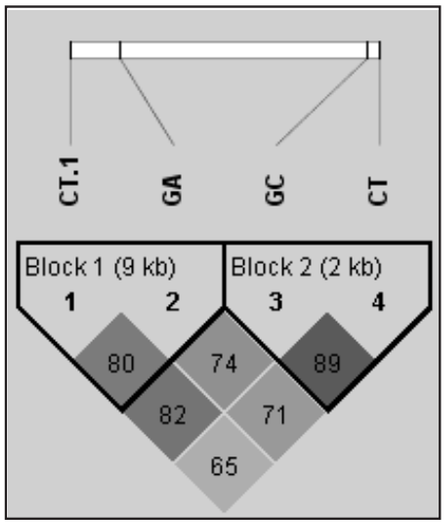
Linkage disequilibrium pattern for four SNPs (rs2203877; rs6265; rs988748; rs2030324) of the BDNF gene was shown on Fig. 1. Analysed SNPs formed two blocks. In the first block we found that D' value is suggestive for linkage disequilibrium between the two of BDNF SNP, namely: C/T and G/A (D'=0.804, LOD=2.12, r2=0.009), but we did not observe any significant differences in haplotype frequencies between patients and controls.

In the second block D' value suggested linkage disequilibrium between two of SNPs, namely: C/G and C/T (D'=0.895, LOD=67.35, r2=0.256). Haplotype GC (rs988748/rs203024) was significantly more frequent in patients than in the controls (p=0.006).

The genotype distribution and allele frequencies of the three selected SNPs (rs1187326; rs1187327; rs2289656) of the NTRK2 gene in patients with BPD and the control group were shown in Tab. 5 (*next page*). We observed differences in distribution of genotypes and alleles in case of the rs2289656 SNP polymorphism of the NTRK2 gene (BPD: p=0.017 for alleles; BPDII: p=0.004 for genotypes, p=0.004 for alleles). The T allele was more frequent in the whole group of patients and in the BPDII subgroups in comparison to the control group. However, this difference was not significant after adjusting the significance level for multiple tests (Tab. 5).

When analysing groups according to gender, we also observed a difference in allele and genotype distribution between patients and healthy controls in the male subgroup diagnosed with BPD, in particular for BPDI (BPD: p=0.013 for genotypes, p=0.004 for alleles; BPDI: p=0.018 for genotypes; p=0.009 for alleles). In the female subgroup, we found such differences only in patients with BPDII (p=0.031 for genotypes; p=0.029 for alleles) but without statistical significance, after the Bonferroni correction (Tab. 6, *next page*).

Figure 1. Linkage disequilibrium map for four BDNF SNPs.
CT.1 - rs 2203877 (C/T); GA - rs 6265 (G/A); GC - rs 988748(C/G); CT - rs 2030324 (C/T)



	Block1D'=0.804, LOD=2.12, r2=0.009				
(9 kb)	Haplotype freq.		Case,Control Frequencies		Chi Square P Value
	CG	0.762	0.775, 0.754	1.212	0.270
	CA	0.180	0.172, 0.186	0.673	0.412
	TG	0.056	0.051, 0.059	0.662	0.415
	Block2	D'=0.895, LOD=67.35, r2=0.256			
(2 kb)	Haplotype freq.		Case,Control Frequencies		Chi Square P Value
	CC	0.529	0.526, 0.531	0.071	0.790
	CT	0.257	0.261, 0.254	0.143	0.705
	GT	0.202	0.193, 0.208	0.686	0.407
	GC	0.012	0.020, 0.006	7.523	0.006

Table 5. Genotype and allele frequencies of polymorphisms of the NTRK2 gene

	n	genotype			p	allele		p	OR (95% IC)
rs1187326		T/T	T/C	C/C		T	C		
control	588	331 (56.3)	226 (38.4)	31 (5.3)		888 (75.5)	288 (24.5)		
Total BPD	454	280 (61.7)	157 (34.6)	17 (3.7)	0.165	717 (78.4)	191 (21.0)	0.070	
Bipolar I	368	223 (60.6)	131 (35.6)	14 (3.8)	0.325	577(78.4)	159 (21.6)	0.134	
Bipolar II	86	57 (66.3)	26 (30.2)	3 (3.5)	0.209	140 (81.4)	32 (18.6)	0.085	
	n	genotype			p	allele		p	
rs2289656		C/C	C/T	T/T		C	T		
control	586	401 (68.4)	170 (29.0)	15 (5.6)		972 (82.9)	200 (17.1)		
Total BPD	451	278 (61.6)	154 (34.2)	19 (4.2)	**0.048**	710 (78.7)	192 (21.3)	0.017	1.31 (1.05-1.63)
Bipolar I	366	235 (64.2)	115 (31.4)	16 (4.4)	0.191	585 (79.9)	147 (20.9)	0.099	
Bipolar II	85	43 (50.6)	39 (45.9)	3 (3.5)	**0.004**	125 (73.5)	45 (26.5)	0.004	1.75 (1.20-2.54)
	n	genotype			p	allele		p	
rs1187327		A/A	A/G	G/G		A	G		
control	586	108 (18.4)	288 (49.2)	190 (32.4)		504 (43.0)	668 (57.0)		
Total BPD	442	74 (16.7)	213 (48.2)	155 (35.0)	0.613	369 (41.4)	523 (58.6)	0.483	
Bipolar I	363	65 (17.9)	171 (47.1)	127 (35.0)	0.716	301 (41.5)	425 (58.5)	0.534	
Bipolar II	79	9 (11.4)	42 (53.2)	28 (35.4)	0.304	60 (37.9)	98 (62.1)	0.264	

Numbers in brackets indicate percentages.
Significance level for multiple tests p=0.0007

Table 6. P value for comparison of genotype and allele distribution of each NTRK2 polymorphism according to gender.

	n	male		n	female	
		genotype	allele		genotype	allele
rs1187326						
		T/T T/C C/C	T&C		T/T T/C C/C	T&C
control	232			355		
Total BPD	187	0.054	0.324	267	0.281	0.132
Bipolar I	156	0.672	0.438	212	0.461	0.244
Bipolar II	31	0.625	0.430	55	0.326	0.183
rs2289656						
		C/C C/T T/T	C&T		C/C C/T T/T	C&T
control	232			353		
Total BPD	186	**0.013**	**0.004**	265	0.573	0.717
Bipolar I	156	**0.018**	**0.009**	210	0.980	0.694
Bipolar II	30	0.180	0.086	55	**0.031**	**0.029**
rs1187327						
		A/A A/G G/G	A&G		A/A A/G G/G	A&G
control	231			354		
Total BPD	183	0.491	0.354	259	0.774	0.683
Bipolar I	154	0.689	0.454	209	0.709	0.901
Bipolar II	29	0.341	0.400	50	0.653	0.450

Significance level for multiple tests p=0.0001

After analysing the data according to the age of onset, the genotype A/A of the NTRK2 gene (rs1187327) was found to be more frequent in the patients with BPDI than in the control group (p=0.033) - data not shown) but with no significance after the Bonferroni correction.

After dividing patients in subgroups according to family history of bipolar disorders, T/T genotype and T allele of the T/C polymorphism of the NTRK2 gene (rs1187326) were more frequent in the subgroup of patients with a positive family history (p=0.035 for genotypes, p=0.016 for alleles – data not shown) but the significance disappeared after the Bonferroni correction.

Analysis performed in a subgroup of patients with a history of suicidal attempts did not show any significant differences in genotype or allele frequency for any of the analysed polymorphisms of the NTRK2 gene (data not shown).

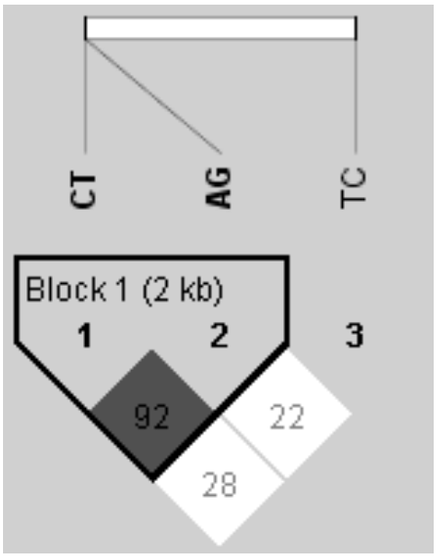
In the linkage disequilibrium analysis of the three SNPs of NTRK2, we found that the D' value is suggestive of a linkage disequilibrium between two of them, namely T/C and A/G (D'=0.926, LOD=105.46, r2=0.351) – Fig. 2. Haplotype CA (rs1187326/rs1187327) was significantly more frequent in patients than in controls (p=0.046).

The power for empirically estimated relative risk (~1.0) for bipolar disorder for each allele in our sample was evaluated as follows: for the SNP`s of BDNF gene (rs2030324 – 5% , rs988748 – 3%, rs6265(Val66Met) – 8%, rs2203877 – 89%) and for the SNP`s of the NTRK2 gene (rs1187326 – 19%, rs2289656 – 39%, rs1187327 – 7%).

DISCUSSION

The main findings of this study, focusing on the possible relationship between bipolar disorder and BDNF and NTRK2 genes, is suggestive evidence for linkage between rs988748 T/C and rs203024 C/T of BDNF gene and rs1187326 T/C and rs118727 A/G of NTRK2 gene. For the two linked polymorphisms of the BDNF gene, one haplotype block was created with the GC combination significant more frequent in the BPD patients, which may suggest a predisposing role of this haplotype in regard to the disease. In case of the NTRK2 gene, haplotype block was created with CA combination significant more frequent in the BPD patients.

Figure 2. Linkage disequilibrium map for four BDNF SNPs
CT.1 - rs 2203877 (C/T); GA - rs 6265 (G/A); GC - rs 988748(C/G); CT - rs 2030324 (C/T)



Block1D'=0.926, LOD=105.46, r^2=0.351

2 kb	Haplotype	Freq.	Case,	Control	Frequencies	Chi Square
	TG	0.569	0.580,	0.561	0.719	0.396
	CA	0.220	0.199,	0.236	3.951	0.046
	TA	0.201	0.211,	0.194	1.0	0.317

However, in our study we did not find any significant association between four selected single nucleotide polymorphisms of the BDNF gene and bipolar affective disorder, neither in the whole group of patients nor in the subgroups based on gender, family history or suicidal attempts. When we divided our sample into subgroups by age of onset (BP age of onset <18 n= 54), we found that Met/Met genotype of the BDNF gene occurred significantly more

frequent in the subgroup of BPDI patients with an early age of onset (≤18 years) than in controls (p=0.034). However, the difference did not reach significance after applying the Bonferroni correction.

Our results may suggest that the single marker of the BDNF gene does not play a major role in the susceptibility to bipolar disorder in this Polish sample which is concordant with the results presented by Zai et al. in 2007, in a Canadian population [41]. The results obtained in this analysis are also consistent with the previous large case–control study of over 3000 individuals from the UK [42], where no association with bipolar affective disorder was found. However, an association was found in case of individuals with rapid cycling BD, but it was limited to the Val66Met polymorphism. It is difficult to discuss the results in the context of previous BDNF association studies for bipolar disorder because their authors performed the analysis using different methods: TDT [43], case-control study where results suggest that the Val allele increases risk for BPI in patients [44], haplotype analysis where the Val/GT(n)short haplotype of BDNF was associated with childhood-onset mood disorders [45]), tested different subgroups of patients (children with bipolar parents [46], rapid cycling BD [42]), different types of polymorphisms VNTR [45]), and different origin populations (Japan [47] and Scotland [48]).

Lack of evident associations throughout the studies in bipolar disorder may result from its heterogeneity. Thus endophenotype analysis is an alternative method for measuring phenotypic variation that may facilitate the identification of gene susceptibility. In our previous study, an association between Val66Met BDNF polymorphism and cognitive performance on prefrontal cortex in bipolar patients was found. Significantly better results on three domains of the WCST were obtained by bipolar patients with Val/Val genotype of Val66Met [49]. In pharmacogenetic analysis of rs6265 polymorphism, the Met66 allele was significantly more frequent in excellent and partial lithium responders in comparison to the non-responders. Similarly analysis of the rs988748 C/G SNP polymorphism of the BDNF gene showed an association with G allele in the partial lithium responders vs. non-responders [32]

Thus, further studies are required to assess the possible association of the BDNF gene polymorphisms with specific diagnostic and phenotypic subgroups of bipolar disorder. Moreover, it cannot be excluded that other, yet unidentified BDNF polymorphisms exist that may be involved in the aetiology of bipolar affective disorder.

In single marker analysis of NTRK2 gene, the main finding is a possible association of the T allele of the NTRK2 polymorphism (rs22899656) with bipolar disorder in the whole group of patients and in the subgroup of patients with type II bipolar disorder. After dividing the groups by gender, T allele was more frequently observed in male patients with a BPDI diagnosis. It is possible that there are gender differences in the genetic background of bipolar disorder and that gender-specific effects (e.g. testosterone, oestrogen, etc.) may play role in the different susceptibility to the type of disease observed in men and women. Other studies performed by our group, including two polymorphisms of MAO-A and 5HTT genes, also demonstrated gender differences, showing an association of those polymorphisms with bipolar disorder only in female patients [50, 51].

Carriers of the A/A genotype of rs1187327 were significantly more often diagnosed with BPDI age of onset of disease <18 (p=0.033). Thus, it may be possible that A/A genotype may be involved in the predisposition to the earlier manifestation of this illness.

In the subgroup of patients with a family history of BPD (n=113) T allele of NTRK2 gene polymorphism (rs1187326) was more frequent than in controls (p=0.016). In our study, the prevalence of family history positive individuals is rather low in comparison with the published data. Obtaining reliable information from patients may be the cause of this discrepancy. Although the social perception of having a family member with a psychiatric illness is still not readily accepted. Moreover, knowledge of a complete family history is rather low and verifying information is difficult. Two of the three polymorphisms of the NTRK2 gene which were analysed in our study had been previously tested in Childhood-Onset Mood Disorder (COMD), by Adams et al. (2005) [52]. They did not find any significant difference in either allele or genotype frequencies be-

tween cases and controls (rs1187362, rs1187327*, rs2289656*; rs* - analysed in this paper). They did not show any evidence of an allelic association for each marker individually or when haplotypes were analysed in the family based sample (n=113 families), using the transmission disequilibrium test (TDT). However, the group they tested was rather heterogeneous. They included patients with depressive disorder and/or dysthymic disorder and bipolar spectrum disorders (BPDI, BPDII and cyclothymic disorder).

There are few studies on NTRK2 polymorphisms in other psychiatric disorders, and those papers presented rather conflicting data. A study on Alzheimer's Disease (AD) of fourteen SNPs remaining in linkage disequilibrium (LD) demonstrated a lack of association in a family-based association test while a significant correlation was found for haplotypes containing SNPs: rs1624327, rs1443445 and rs378645 [53]. In a study of 14 NTRK2 SNP polymorphisms in Eating Disorders (ED) conducted by Rebases et al. (2005) in a group of 164 Spanish patients, a significant overrepresentation of the C/C genotype (rs1187325; -69C/G) and the G/G genotype (rs1047896; 40G/A) in binge-eating/purging patients have been reported [54]. The authors also showed an association of the C/A/insC haplotype with an anorexia nervosa binge-eating/purging subtype of the disease and a high score for harm avoidance (p=0.006; p=0.003, respectively) (rs1187325; -69C/G, rs1047896; 40G/A, rs10123741; 2784-2785insC). Polymorphisms rs1187326 (C/T) and rs2289656 (G/A), analysed in this paper, did not show any significant relationship either in the classical association test nor in the haplotype analysis with ED. Recently, in a study on Obsessive-Compulsive Disorder (OCD), Alonso et al. (2007) detected a significant association of the intronic SNP (rs238672) with OCD ($p<0.0001$), but in female patients only [55]. Recently published data from genome-wide association (GWA) studies indicated strong evidence for association of not previously tested rs2769605 in the NTRK2 gene with BPD [56]. This indicates that all possible SNPs need to be identified and a detailed analysis has to be provided to indicate which of these polymorphisms may be involved in predisposition to BPD. Unfortunately, this is not a straightforward task, particularly because of the complicated structure of the NTRK2 gene and the fact that the functional significance of numerous SNPs of the NTRK2 gene is still not known.

The main limitation of this study is the low power of analysed polymorphisms. Power analysis helps to estimate if the number of subjects in the analysed groups were sufficient to predict positive association. It is not surprising that in complex diseases, power estimation very often does not exceed 10%. Therefore, usually the results obtained in this study should be interpreted very carefully and this indicates the need for replication studies on larger groups.

The second limitation in our study is lack of psychiatric screening in the control subjects. However, the possibility of including subjects with psychiatric disorders into the control group is relatively low, because the prevalence of psychiatric disorders is 3% in the general population.

Single marker case-control analysis provided for BDNF and NTRK2 genes did not show any significant results after applying correction for multiple tests. However, the strength of our study is that positive association of BDNF and NTRK2 haplotype has been demonstrated in relatively large groups.

In conclusion, our results suggest that BDNF and NTRK2 genes may be susceptibility genes contributing to BPD pathophysiology in the studied population, but taking into account the limitations of the present study, these results need to be replicated before reaching a final conclusion.

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