Short Communication

Polymorphisms in Serotonin-Related Genes in Anorexia Nervosa. The First Study in Czech Population and Meta-analyses with Previously Performed Studies

(anorexia nervosa / gene polymorphisms / serotonin / meta-analysis / Czech population)

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Abstract. Anorexia nervosa is a serious psychiatric disorder characterized by the inability to maintain normal body weight. The frequently studied polymorphisms in the serotonin *5-HT2A* receptor gene (-1438A/G) and in serotonin transporter *5-HTT* gene (LPR, VNTR) have led to controversial results in different populations. The aim of the study was to address association of the above-mentioned polymorphisms with anorexia nervosa in the Czech population. We genotyped a well-defined group of 75 patients with anorexia nervosa (average age of 25.39 years, SD 6.18; average BMI 14.65 (SD 1.38)). The control group consisted of 65 Caucasian healthy females (average age 25.76 years, SD 5.12; average BMI 20.69, SD 1.85). The *5-HT2A* receptor -1438A/G polymorphism analysis showed a trend for the association with odds ratios for risk allele A being in the same direction. In combination with a previously published Polish cohort, the allelic test reached a suggestive borderline (P = 0.0362, χ^2 statistics, 1 df). In meta-analysis which included all published results for allelic tests, the resulting P value was highly significant (0.0003, χ^2 statistics, 1 df). Using quantitative association of *5-HTR2A* polymorphism with BMI in the Czech sample, a borderline association (P = 0.055) was observed. In *5-HTT*, LPR polymorphism analysis, unlike in *5-HT2A*, neither allelic nor quantitative association with BMI for the bi-allelic *5-HTT* marker was observed. Results of this study support previous reports of a significant role of the A allele (-1438A/G, 5-HT2A receptor) as a risk factor in anorexia nervosa.

Introduction

Anorexia nervosa (AN) represents the most serious psychiatric disorder from the spectrum of eating disorders (ED) characterized by a pathological eating pattern with an inability to maintain a normal, healthy body weight. AN predominantly occurs in women and is characterized by restricted eating and purging behaviour, obsessive fears of becoming overweight, pathological body image perception, and other multiple medical, hormonal, psychological and social complications (Faiburn and Harrison, 2003). Weight is maintained at least 15 % below that expected (either lost or never achieved), or Quetelet's body mass index (BMI) is 17.5 or less. Prepubertal patients fail to make the expected weight gain during the period of growth. The weight loss is self-induced by diets, avoidance of "fattening foods" and one or more of the following: self-induced vomiting, self-induced purging, excessive exercise, use of appetite suppressant and/or diuretics.

The body image distortion observed in AN patients is in the form of a specific psychopathology: with increasing emaciation, the patient's feeling of being too large persists and she imposes upon herself a low weight threshold. AN is an endocrine disorder of the hypothalamic-pituitary-gonadal axis, characterized by amenorrhea in women frequently "masked" by hormonal replacement therapy and in men by loss of sexual interest and potency. Elevated levels of growth hormone and cortisol, as well as decrease in thyroidal hormone and abnormalities in insulin secretion are also typical, as is onset-delayed or stopped development (growth, breasts and the genitals) in prepubertal patients (International

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Abbreviations: 5-HT – serotonin, 5-hydroxytryptamin, AN – anorexia nervosa, BMI – body mass index, ED – eating disorders, LPR – linked polymorphic region, OR – odds ratio, VNTR – variable number of tandem repeats.

Statistical Classification -10, 2004, Diagnostic and Statistical Manual of Mental Disorders -IV-TM, 2000).

Meta-analysis of 42 outcome studies showed an annual mortality rate of 0.56 %; 12 times higher than in the general population (Sullivan, 1995). The risk of death from eating disorders was described to be three times higher than in depression, schizophrenia or alcoholism (Harris and Barraclough, 1998).

The pathogenesis of AN is multifactorial with a clear genetic component (Sulek et al., 2007; Mazzeo et al., 2009). The frequently studied polymorphisms in the serotonin receptor (-1438A/G) and transporter (*5-HTT* linked polymorphic region (LPR)) genes have led to controversial results (Serretti et al., 2007). However, other fields of genetic research in ED showed that appetite homeostasis (Hebebrand and Remschmidt, 1995, Krizova et al. 2008) and obsessive compulsive personality disorder (OCPD) temperamental traits (Lilenfeld et al., 1998) contributing to development of the disease are under genetic control. Serotonin (5-hydroxytryptamine; 5-HT) may play an important role in these mechanisms. Serotonin serves as an important neurotransmitter in the central as well as peripheral nervous systems. This neurotransmitter occupies a unique place in neurobiology, playing an important role in many physiologic processes – sleep, pain perception, appetite, hormone secretion, sexual behaviour, and thermoregulation (Kaye, 2008; Murphy et al., 2008).

There are lines of evidence associating changes in serotonergic activity with vulnerability to abnormal eating behaviour and in the pathogenesis of AN. The frequently studied polymorphisms in the promoter of serotonin *5-HT2A* receptor gene (-1438A/G) and in the serotonin transporter (*5-HTT* LPR, variable number of tandem repeats (VNTR)) gene have led to controversial results in different populations (Gorwood et al., 2003). Therefore, the aim of the study was to genotype a well-defined group of patients with AN, and respective controls, to address the association of the above-mentioned polymorphisms with AN in the Czech population. Such a study has not been performed to date.

Material and Methods

Probands

The study was approved by the Institutional Ethics Committee and all patients and controls provided their written informed consent. We genotyped 75 (for *5-HT2A*) and 72 (for *5-HTT*) patients with AN (diagnosed with DSM-IV and ICD-10, American Psychiatric Association) during their hospitalization. The BMI was calculated to be 14.65 on average (SD 1.38, range from 10.35 to 17.96), and the average age was 25.39 years (SD 6.18, range from 18 to 47). The control group consisted of 65 Caucasian healthy females, mostly students from regions of Bohemia and Moravia, Czech Republic, similar to the areas from which the patients came, the average age was 25.76 years (SD 5.12, range from 17 to 43) and average BMI was 20.69 (SD 1.85, range from 17.64 to 28.68).

Genomic DNA preparation

Genomic DNA was extracted from peripheral blood anticoagulated with EDTA according to a standard protocol.

5-HT2A receptor -1438 polymorphism genotyping

Polymorphism -1438A/G in the promoter of the gene for the 5-HT2A receptor was analysed after DNA amplification with PP Master mix polymerase from Top-Bio Ltd., Prague, Czech Republic (initial denaturation 95 °C/2 min followed by 30 cycles of 95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; with final extension at 72 °C for 2 min) using following oligonucleotides:
1438G/A Fw: 5‘-AAGCTGCAAGGTAGCAACAGC-3‘
1438G/A Rev: 5‘-AACCAACTTATTTCCTACCAC-3‘

The resulting DNA product was digested using *Msp*I. In the presence of the -1438G allele, digestion led to two fragments of 244 bases and 224 bases, respectively. In the presence of the -1438A allele the resulting product of amplification (468 bases) was not digested. The resulting products were separated using 1.5% agarose (Collier at al., 1997; Ricca at al., 2004).

5-HTT genotyping for 5-HTT LPR and 5-HTT VNTR polymorphisms

The short and long alleles of *5-HTT* LPR were amplified using primers
LPR Fw: 5′-GGCGTTGCCGCTCTGAATG-3′ and
LPR Rev: 5′-GAGGGACTGAGCTGGACAACCAC-3′.

The *5-HTT* VNTR region was amplified using primers:
VNTR Fw: 5′-GCTGTGGACCTGGGCAATGT-3′ and
VNTR Rev: 5′-AGTGAAGACTGAAAAGACATAATC-3′,

which yielded fragments containing 12 (STin2.12), 10 (STin2.10) or 9 (STin2.9) copies of the repeat element.

The PCR reactions were carried out in a total volume of 25 μl, including 50 ng of genomic DNA, 0.4 mM of each primer, and 1x Plain PP Master Mix: 150 mM Tris-HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 μM dATP, 400 μM dCTP, 400 μM dGTP, 400 μM dTTP, and 100 U/ml Taq DNA polymerase (Top-Bio Ltd.). Initial denaturation was at 94 °C for 2 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 65 °C (*5-HTT* LPR) or 63 °C (*5-HTT* VNTR), and 45 s elongation at 72 °C, with a final extension at 72 °C for 5 min. The PCR products were separated by 8% PAGE and stained with ethidium bromide (modified according to Betancur et al., 2002).

Statistical analysis

Allelic test, full association model and quantitative association test (Wald test) for bi-allelic markers (*5-HT2A*, *5-HTT* LPR) were calculated using routine procedures

Table 1. Frequency of genotypes and alleles of -1438A/G polymorphism in the 5-HT2A gene in patients and healthy controls

	Genotypes			Alleles	
	A/A	G/A	G/G	A	G
Patients	16 (21.3)	39 (52.0)	20 (26.7)	71 (47.3)	79 (52.7)
Controls	11 (16.9)	33 (50.8)	21 (32.3)	55 (42.3)	75 (57.7)

Table 2. Frequency of genotypes and alleles of LPR polymorphism in the 5-HTT gene in patients and healthy controls

	Genotypes			Alleles	
	S/S	L/S	L/L	S	L
Patients	12 (16.7)	29 (40.3)	31 (43.0)	53 (36.8)	91 (63.2)
Controls	13 (20.0)	30 (46.1)	22 (33.9)	56 (43.1)	74 (56.9)

Table 3. Frequency of genotypes of VNTR polymorphism in the 5-HTT gene in patients and healthy controls

	Genotypes					
	9/9	9/10	9/12	10/10	10/12	12/12
Patients	0	3 (4.6)	4 (6.1)	8 (12.1)	28 (42.4)	23 (34.9)
Controls	0	0	1 (1.4)	14 (19.7)	32 (45.1)	24 (33.8)

Table 4. Frequency of alleles of VNTR polymorphism in the 5-HTT gene in patients and healthy controls

	Alleles		
	9	10	12
Patients	7 (4.8)	59 (41.0)	78 (54.2)
Controls	1 (0.8)	48 (36.9)	81 (62.3)

incorporated in the PLINK statistical software package (Purcell et al., 2007). The association test for multiallelic marker *5-HTT* VNTR was calculated with UNPHASED statistical suite (Dudbridge, 2003). The standard χ^2 statistics with one degree of freedom (df) were calculated in STATISTICA 99 (StatSoft Inc., Tulsa, OK, http://www.statsoft.com). Individual odds ratios (ORs) were calculated using a web-based calculator (Bland and Altman, 2000), and for statistical power estimation we also used a web-based application – Genetic Power Calculator (Purcell et al., 2003).

Results and Discussion

Frequency, genotypes and alleles of three serotonin-related polymorphisms investigated in this study are depicted in Table 1 to Table 4.

5-HT2A, -1438A/G: The statistical power of the Czech cohort with entrance parameters based on previous reports is 8–9 %. In this cohort only, there is a trend for the association with OR for risk allele A being in the same direction. In combination with a Polish cohort, allelic test reached a suggestive borderline (P = 0.0362, χ^2 statistics, 1 df) (Rybakowski et al., 2006). Once all published results for allelic tests were taken together, the resulting P value was highly significant (0.0003, χ^2 statistics, 1 df). Results of meta-analysis for -1438A/G polymorphism of *5-HT2A* are shown in Table 5.

By including previously published AN results a remarkable increase of statistical significance was observed thanks to a sufficient sample size. However, heterogeneity of the population and thus the possible effect of stratification within these populations has to be taken into account. Also, using quantitative association of the *5-HT2A* polymorphism with BMI in the Czech sample, a result with borderline association (P = 0.055) was observed (Table 6).

The role of the -1438A/G polymorphism in *5-HT2A* in AN in different ethnic groups was repeatedly investigated in an attempt to replicate the classic study of Collier at al. (1997), who found in the British cohort of AN patients 51 % having the -1438A allele while the in control group it was only 42 %, a statistically significant difference. A similar trend was found in the following study with another British cohort (Campbell et al., 1998; 48 % vs. 42 %). A higher proportion of the -1438A allele among AN patients was also found in two independent Italian cohorts (Sorbi et al. 1998, 56 % vs. 36 %; Nacmias et al., 1999, 55 % vs. 39 %) and a cohort from the United States (Enoch et al., 1998; 51 % vs. 36 %). In the only study addressing Slavic population with AN in Poland, the -1438A allele appeared in 65 % in patients while it was found in 57 % of controls. The results with opposite tendency were found in two independent German studies (Hinney at al., 1997a,b, 40 % vs. 42 %; Ziegler et al., 1999, 36 % vs. 34 %), a French study (Kipman et al., 2002, 42 % vs. 48 %) and a Japanese study (Nishiguchi et al., 2001, 46 % vs. 54 %). Our results shows allele -1438A in 47 % patients with AN and in 42 % of controls (Table 1).

5-HTT LPR and *5-HTT* VNTR**:** Unlike in the other marker, *5-HT2A*, we have observed neither allelic nor quantitative association with BMI for the bi-allelic *5-HTT* LPR marker. The odds ratios in the Czech sample displayed an opposite trend than in all other published studies, and their inclusion into the combined analysis lowered the statistical significance and OR, and broadened its 95% confidence interval (Table 7). In the

Table 5. Meta-analysis of the association studies analysing the -1438A allele of the 5-HT2A gene in anorexia nervosa patients and controls. ORs are shown with their 95% confidence intervals. ([A]Rybakowski et al., 2006; [B]Gorwood et al., 2003)

Origin	Group	A allele	G allele	P	OR	95% CI from	95% CI to
Czechs	Patients	71	79	0.3992	1.2255	0.7636	1.9668
	Controls	55	75				
Poles[A]	Patients	170	92	0.0847	1.4087	0.9535	2.8110
	Controls	101	77				
Slaves (Czechs + Poles)	Patients	241	171	0.0362	1.3732	1.0202	1.8484
	Controls	156	152				
Nine previously analysed cohorts[B]	Patients	816	928	0.0043	1.2000	1.0700	1.3500
	Controls	1443	1869				
Nine previously analysed cohorts + Slaves	Patients	1057	1099	0.0003	1.2156	1.0923	1.3528
	Controls	1599	2021				

Table 6. Quantitative association with BMI (Wald test)

Polymorphism	NMISS	BETA	SE	R2	T	P
5-HT2A	119	-0.9076	0.4696	0.030940	-1.933	0.05569
5-HTT LPR	119	-0.4717	0.4500	0.009304	-1.048	0.29670

Table 7. Meta-analysis of the association studies analysing the S and L alleles of the 5-HTT gene in anorexia nervosa patients and controls. ORs are shown with their 95% confidence intervals. ([A]Rybakowski et al., 2006; [C]Gorwood et al., 2004)

Origin	Group	S allele	L allele	P	OR	95% CI from	95% CI to
Czechs	Patients	53	91	0.2896	0.7696	0.4738	1.2499
	Controls	56	74				
Poles[A]	Patients	78	114	0.4170	1.1873	0.7842	1.7975
	Controls	68	118				
Slaves (Czechs + Poles)	Patients	131	205	0.9474	0.9895	0.7223	1.3555
	Controls	124	192				
Four previously analysed cohorts[C]	Patients	341	373	0.0009	1.3800	1.1400	1.6800
	Controls	374	566				
All cohorts combined	Controls	472	578	0.0102	1.2430	1.0530	1.4680
	Controls	498	758				

multiallelic *5-HTT* VNTR polymorphism, we have not observed any association as well.

In the study of Estonian adolescent girls it was shown that homozygozity in the *5-HTT* LPR long allele, indicator of higher serotonin system capacity, indicated a higher drive for thinnes (Akkermann et al., 2008).

Recently, the new interest for genotyping of the *5-HT2A* gene variants showed, additionally to the role in AN, a possible role of these polymorphisms in antidepressant pharmacogenetics (Ramoz et al., 2007; Benedetti et al., 2008; Monteleone and Maj, 2008), as well as in the predisposition to obesity in different geographic areas (Sorlí et al., 2008; Ying et al., 2009).

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