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ASSOCIATION OF THE GLUCOCORTICOID RECEPTOR GENE POLYMORPHISMS AND THEIR INTERACTION WITH STRESSFUL LIFE EVENTS IN POLISH ADOLESCENT GIRLS WITH ANOREXIA NERVOSA

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SUMMARY

Background: *Disturbances in stress response mechanisms and hypothalamic-pituitary-adrenal axis (HPA) functioning are considered important factors involved in the pathophysiology of anorexia nervosa (AN). Thus, genetic variations in the end effector of HPA - glucocorticoid receptor gene and relationships to stressful life events (SLE) may be connected to a higher risk of illness. The aim of the study was examining the association between glucocorticoid receptor gene (NR3C1) polymorphisms and risk factors among stressful life events in AN patients.*

Subjects and methods: *This study comprised 256 patients with AN and 167 control subjects. The questionnaires examining brief history of the mother's pregnancy and long-acting stress factors, as well as life events checklist to assess stressful life events during the 6 months prior to hospitalization were used. The eight common SNPs (rs6198, rs6191, rs6196, rs258813, rs33388, rs41423247, rs56149945 and rs10052957) of NR3C1 gene were genotyped.*

Results: *The association of five polymorphisms (rs6191, rs258813, rs33388, rs41423247 and rs10052957) and one complex allele (TCAGT) of NR3C1 gene with increased risk of AN were found. However, no significant correlations between early, long-acting and predicting hospitalization SLE and any of the analyzed polymorphisms were observed.*

Conclusions: *The results confirm that the NR3C1 gene is associated with AN risk regardless of the type of stressful triggering factors.*

Key words: *anorexia nervosa - glucocorticoid receptor gene - NR3C1 gene - stressful life events*

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INTRODUCTION

The participation of both genetic and environmental factors have been postulated in anorexia nervosa (AN) etiology (Helder & Collier 2011, Trace et al. 2013). Kendler & Prescott (2006) define genetic and environmental interactions (GxE) as the differential expression of genetic predisposition in the different environmental conditions (Kendler & Prescott 2006). This means that disease symptoms could be due to environmental factors actions, which exceed the genetically determined buffering capabilities of the organism. Trace at al. indicates that G×E interactions are particularly relevant to the study of eating disorders and it is future directions in eating disorders and genetics research (Trace et al. 2013). Long-acting stress factors, particularly during childhood (e.g., loss of parents or sexual violence) are among the major AN risk factors (Palazzoli 1996), and stressful life events, such as school transition or breaking up with a partner later in life, can trigger a genetic predisposition to the development of anorexia (Berge et al. 2012). At the molecular level, the hypothalamic-pituitary-adrenal axis (HPA) is an important coupling mechanism between stress factors and the disturbed outcomes observed in AN patients. Indeed, according to the neurodevelopmental model of anorexia nervosa (AN) proposed by Connan et al. (2003), abnormalities in stress response mechanisms and in the functioning of the HPA axis could be important factors involved in the pathophysiology of AN (Connan et al. 2003). There is also evidence that cortisol levels are elevated in AN patients (Lo Sauro et al. 2008). In addition, impairment of the inhibitory effect of the dexamethasone suppression test reveal a flaw in the negative feedback mechanism in AN (Putignano et al. 2001). Furthermore, Anacker and others have demonstrated that both stress and glucocorticoid pathway activation consistently decrease hippocampal neurogenesis in vitro and in vivo (Anacker et al. 2013). These findings provide evidence in favor of a neurodevelopmental model of AN. The glucocorticoid receptor (GR) encoded by the NR3C1 gene is the end effector of HPA action and plays a crucial role in glucocorticoid signaling; see review in (Briassoulis et al. 2011) and (Ising & Holsboer 2006, Tsigos & Chrousos 2002). Animal studies show that polymorphisms in this gene

can influence the normal basal activity of the HPA axis, with increased stress-induced corticosteroid levels and impaired negative feedback (Kaye 2008). Thus, genetic variations in the glucocorticoid receptor gene NR3C1 may affect both the expression and properties of the receptor protein, suggesting a role in AN. However, only one study to date has focused on AN, though several associations between psychiatric disorders have appeared (Cellini et al. 2010). We conducted a case-control analysis as the first step in the present study and then assessed complex alleles or SNP interactions between NR3C1 polymorphisms that conferred AN risk. We next analyzed the relationship between NR3C1 gene variants with 1) stressful life factors existing in the perinatal period, 2) long-acting stress factors during childhood and 3) potential trigger factors that occurred during the six months prior to hospitalization.

SUBJECTS AND METHODS

All of the patients and healthy controls involved were females of Polish origin. We recruited the patients (n=256; mean age 17.5+/-3.3) from inpatients treated in the Department of Child and Adolescent Psychiatry of Poznan University of Medical Sciences. Anorexia nervosa was diagnosed according to ICD-10 (F.50.0) criteria with structured interview and confirmed by the senior researcher (WHO 1992). Main exclusion criteria were: documented organics injures of central nervous system, serious somatic disorder as well as the use of pharmacotherapy at the time of the study which might affect on the behavior and/or cognitive function. Also from the study were excluded patients who had coexisting schizophrenia and bipolar disorder. In pursuing its objectives the study used a structured clinical interview (age, education, age of onset, BMI). Depression severity was assessed with self-reported Beck Depression Inventory (BDI). We used a modified version of the Life Events Checklist (LEC) adopted for adolescents and young adults to assess stressful life events (SLE) during the 6 months prior to hospitalization (Rietschel 2004). LEC contains 79 life events grouped into 9 domains: relationship, children, changes/separation, health, school/job, finance, law, accommodation and sexuality. Data from 68 patients were obtained. Additionally, we analyzed information obtained from the authors' clinical interview with parents, which included a brief history of the mother's pregnancy (to determine perinatal abnormalities), school, material and family situation and conflict existing in the patient's family as long-acting stress factors; data were obtained from 237 patients.

Control subjects (n=167; mean age 19.6=/-3.2) were recruited among girls attending randomly selected intermediate and high school. Only volunteers neither without eating disorders or other mental disorders nor family history of these disorders among their first-degree relatives were carefully chosen. The information was obtained from the interview filled out by parents. A detailed description of the patient and control groups is presented in Table 1.

Table 1. Description of examined samples

	Anorexia Nervosa patients	Control group
Number of subjects (n)	256	167
Age (years)	17.482±3.341	19.617±3.186
BMI	14.389±1.945	21.469±9.578
BDI	17.500±11.585	6.708±6.830
Education (years)	11.306±2.852	13.509±3.008
Age of onset (years)	14.983±2.842	-
Family history (n) (%)	71 (27.73)	-
SLE (N=237)	1.170±1.440	-
LEC (N=68)	3.868±5.812	-

BMI - Body Mass Index; BDI - Beck Depression Inventory; SLE - Stressful Life Events; LEC - Life Events Checklist

We genotyped 8 SNPs (rs6198/GR9β, rs6191, rs6196/Asn766Asn, rs258813, rs33388, rs41423247/BclI, rs56149945 previous rs6195/N363S, rs10052957/Tth111I) of NR3C1 gene. Markers selection was based on the previous reports in mood and eating disorders (Cellini et al. 2010, Di Blasio et al. 2003, Galecka et al. 2013, Van Rossum & Lamberts 2004). The genotypes were determined by a previously described method (Szczepankiewicz et al. 2011).

Statistical Analysis

The statistical analyses were performed with licensed statistical package STATISTICA v. 10.0. Also we used Haploview v. 4.2 (Barrett et al. 2005), and QUANTO v. 1.2.4 (Gauderman 2002) software, Genetic association analyses were assessed using R statistical software environment (Team 2011) with its specific package "SNPassoc" (González et al. 2012). We analysed association between alleles and SLE and LEC scores using PLINK software (Purcell et al. 2007). Linear regression models was applied to seek for quantitative trait association (command: p-link -file -linear -adjust). Having no prior assumptions about inheritance patters, we chose additive model as suggested by (Konietschke et al. 2012). We used false discovery rate (FDR) procedure as correction for multiple testing (Benjamini & Hochberg 1995). To detect associations on a genotype level we applied Kreskas-Wallis test. In order to analyze pair wise relations U-Mann test with FDR adjustment.

The study protocol was approved by the Ethics Committee of the Poona University of Medical Sciences. A written consent signed by all the participants or their guardians were obtained.

RESULTS

Table 2 presents the results of the case-control analysis. All of the markers are consistent with Hardy-Weinberg equilibrium. We found a significant nominal association between five polymorphisms (rs6191, rs258813, rs33388, rs41423247 and rs10052957) and an increased risk of AN. However, after multiple testing corrections, only two polymorphisms remained: rs6191 (p=0.014) and rs33388 (p=0.024).

Linkage disequilibrium was observed between five NR3C1 polymorphisms, defining one block (D′>0.95; LOD>70, r2>0.45). We found that the heliotype variants TCAGT created a complex allele of Snips (rs6198, rs6191, rs6196, rs258813 and rs33388) that was associated with an increased susceptibility of AN (nominal p=0.003 and after correction p=0.023).

We performed a two-dimensional analysis of epistatic SNP-SNP interactions using a log-additive model (González et al. 2012), though no significant interaction AN model was identified (p>0.080) (data not shown).

In the overwhelming group of patients pre- and postnatal development was within the norm. However, in individual cases, 1) at-risk pregnancy (n=16), 2) premature or delayed birth (n=29) and 3) assisted labor or Caesarean section (n=34) were identified. Only 11 patients were born with a low birth weight (<2,500 g), mainly due to premature birth. A low Apgar scale (1-3) score was found in two cases, suggesting perinatal hypoxia.

Learning problems requiring repetition of a year in school was found in two patients. A total of 47 patients came from single-parent, broken or reconstructed families, and 22 parents rated their financial condition as bad or very bad.

The most common cause of conflict in the family was alcohol abuse (n=67), leading to conflicts and a poor economic situation. Among other problems, the most commonly reported by the patients included a lack of acceptance (n=13), parent separation (n=19), parent or sibling death (n=14) and violence (n=8).

The patients participating in the study were questioned about the circumstances preceding the current episode of illness (six months before the current episode). The events declared were assigned to the following categories: relationship (n=30), changes/ separation (n=22), health (n=22), school/job (n=33), finance (n=6), law (n=3), accommodation (n=8) and sexuality (n=2). The majority of patients reported the following: a lack of understanding from parents (n=17), preparing for final exams (n=17), starting a new school (n=13), first dating (n=12), the hospitalization due to disease (n=12), graduation (n=12) and being humiliated at school or work (n=10). All of the categories were obtained from LEC (Tables 3 & 4).

In addition to genetic association analyses we applied Poisson regression models to evaluate genetic variants and clinical characteristics (BMI or BDI scores) as SLE and LEC predictors. We looked for: 1) main effects (using multiple regression equation: SLE ~ genotype + BMI + BDI) and for GxE interactions (taking genetic variants and BMI, BDI as covariates: SLE number~ genotype*BMI + genotype*BDI).

Computations for SLE and LEC were performed separately, as we collected LEC scores for part of the sample only. We adjusted our models for age. As we recruited only female group exclusively no adjustment for age was necessary. No significant correlations between early, long-acting SLE or LEC predicting hospitalization and any of the analyzed polymorphisms and clinical covariates were observed (data not shown).

Table 2. Comparison of allele and haplotype frequencies for analyzed NR3C1 polymorphisms between AN patients and control group

SNP ID	Associated Allele	MAF	Case, Control Frequencies	χ^2	P value	Permutations	OR (95%CI)
rs6198	T	0.163	0.846, 0.826	0.666	0.414	NS	
rs6191	C	0.475	0.519, 0.417	9.666	*0.001*	*0.014*	*1.509 (1.163-1.956)*
rs6196	A	0.165	0.854, 0.810	3.254	0.071	NS	
rs258813	G	0.323	0.706, 0.639	4.716	*0.029*	NS	
rs33388	T	0.476	0.517, 0.422	8.385	*0.003*	*0.024*	*1.499 (1.516-1.944)*
rs41423247	G	0.360	0.673, 0.596	6.010	*0.014*	NS	
rs56149945	C	0.062	0.941, 0.934	0.203	0.651	NS	
rs10052957	G	0.328	0.705, 0.627	6.243	*0.012*	NS	
SNPs in block	Haplotype variants	Frequency	Case, Control Frequencies	χ^2	P value	Permutations	OR (95%CI)
rs6198 rs6191	*TCAGT*	0.470	0.512, 0.416	8.665	*0.003*	*0.023*	*1.473 (1.234-1.257)*
rs6196	TAAGA	0.200	0.187, 0.217	1.312	0.252	NS	
rs258813	TAGAA	0.160	0.141, 0.185	3.436	0.063	NS	
rs33388	CAAAA	0.159	0.149, 0.172	0.915	0.338	NS	

Table 3. Stressful life events score & mean values

Life Events Checklist	N	Mean	Life Events Checklist	N	Mean
Relationships			Health		
Unrequited love	9	0.13	First amenorrhea	12	0.18
Starting to date	12	0.18	A less serious illness or injury	6	0.08
Serious relationship	7	0.10	An illness or injury with kept you in bed for week or more, or sent you to the hospital	12	0.18
Betrayal of partner	2	0.03			
Breaking up with a close girlfriend or boyfriend (after more than 3 months, not living together)	5	0.07	Chronic somatic disease (resulting in a significant reduction in quality of life)	1	0.01
Serious arguments partner	7	0.10	Chronic pain	4	0.06
Breaking up a relationship (active)	8	0.12	Chronic insomnia	4	0.06
Breaking up a relationship (passive)	5	0.07	Serious illness of close family member	4	0.06
Infidelity of partner	3	0.04	School/work		
A separation from your partner	5	0.07	Starting a new school	13	0.19
The loneliness, the inability to count on help from others	9	0.13	Finishing full-time education	12	0.18
Lack of understanding by parents	17	0.25	Change schools, college or university	5	0.07
Changes/separation			Preparing for a final exam	17	0.25
The birth of an brother or sister	1	0.01	Start new type of work	1	0.01
Having someone new move in with your family/household (grandparents, adopted brother, sister, or other)	2	0.03	A change in work hours and conditions	1	0.01
			A change in responsibilities at work	2	0.03
Permanent break up with important person (e.g., a close friend)	6	0.08	Trouble with teachers, principal, boss, or co-workers	3	0.04
			Fired from work	1	0.01
Serious illness of family member	8	0.12	Unemployment in the family longer than 1 month	4	0.06
Serious argument with a family member (living in the same household))	8	0.12			
			Retirement in the family	1	0.01
Continuous conflict with a family member (living in the same household) (>6 months)	3	0.04	Continuous overwork (>6 months)	8	0.12
			Bullying at school or work (>6 months)	10	0.15
Serious argument with a family member (not living in the same household))	2	0.03	Finanse		
			Moderate financial problems in the family (low income, debts)	4	0.06
Continuous conflict with a family member (not living in the same household) (> 6 months)	2	0.03	Severe financial problems (no income, distraint)	1	0.01
A loss or damage of personal property	1	0.01	Raise or overdraw a credit	1	0.01
Losing a favorite pet	2	0.03	Close friend or relative having severe financial problems	2	0.03
The death of a close family member	6	0.08			
Divorce of parents	3	0.04	Accommodation		
Separation from family	1	0.01	Move within the same town or city	5	0.07
Conflict between parents	9	0.13	Move into a different country	1	0.01
Low			Cramped, stressful living situation/ condition (e.g. lack of privacy in the living quarters, stressful neighborhood (>6 months)	4	0.06
An accident (not your fault)	1	0.01			
An accident (your fault)	1	0.01			
Minor violation of the law (traffic ticket etc...)	1	0.01	Sexuality		
			Sexual dissatisfaction (>6 months)	2	0.03
Testify in court (as a witness)	1	0.01			

DISCUSSION

In the present study, we detected a significant association between NR3C1 polymorphisms and an increased risk of AN, with a relevant relationship between the rs6191, rs33388 and TCAGT haplotype variants (including rs6198 (GR9β), rs6191, rs6196 (Asn766Asn), rs258813 and rs33388) of NR3C1 and AN. The results obtained support previous findings that genetic variants of NR3C1 are associated with AN (Cellini et al. 2010). Our results are also consistent with the possible involvement of variants mentioned in the etiology of depressive

Table 4. Frequency of stressful life events

Lack of understanding by parents	17	Cramped, stressful living situation/condition (e.g. lack of privacy in the living quarters, stressful neighborhood (> 6 months)	4
Preparing for a final exam	17		
Starting a new school	13	Infidelity of partner	3
Starting to date	12	Continuous conflict with a family member (living in the same household) (>6 months)	3
First amenorrhea	12		
An illness or injury with kept you in bed for week or more, or sent you to the hospital	12	Divorce of parents	3
		Trouble with teachers, principal, boss, or co-workers	3
Finishing full-time education	12		
Bullying at school or work (>6 months)	10	Betrayal of partner	2
Unrequited love	9	Having someone new move in with your family/ household (grandparents, adopted brother, sister, or other)	2
The loneliness, the inability to count on help from others	9		
Conflict between parents	9	Serious argument with a family member (not living in the same household))	2
Breaking up a relationship (active)	8		
Serious illness of family member	8	Continuous conflict with a family member (not living in the same household) (>6 months)	2
Serious argument with a family member (living in the same household))	8		
		Losing a favorite pet	2
Continuous overwork (>6 months)	8	A change in responsibilities at work	2
Serious relationship	7	Close friend or relative having severe financial problems	2
Serious arguments partner	7		
Permanent break up with important person (e.g., a close friend)	6	Sexual dissatisfaction (> 6 months)	2
		The birth of an brother or sister	1
The death of a close family member	6	A loss or damage of personal property	1
A less serious illness or injury	6	Separation from family	1
Breaking up with a close girlfriend or boyfriend (after more than 3 months, not living together)	5	Chronic somatic disease (resulting in a significant reduction in quality of life)	1
		Start new type of work	1
Breaking up a relationship (passive)	5	A change in work hours and conditions	1
A separation from your partner	5	Fired from work	1
Change schools, college or university	5	Retirement in the family	1
Move within the same town or city	5	Severe financial problems (no income, distraint)	1
Chronic pain	4	Raise or overdraw a credit	1
Chronic insomnia	4	An accident (not your fault)	1
Serious illness of close family member	4	An accident (your fault)	1
Unemployment in the family longer than 1 month	4	Minor violation of the law (traffic ticket etc ...)	1
		Testify in court (as a witness)	1
Moderate financial problems in the family (low income, debts)	4	Move into a different country	1

disorder (Galecka et al. 2013), major depression and predominance of depression in the course of bipolar disorder in the Polish population (Szczepankiewicz et al. 2011). Depressive symptoms may affect up to 80% of AN patients (Lee et al. 2003). Due to such high the prevalence of depressive symptoms in the course of anorexia nervosa we may treats them as one of the symptoms of AN like BMI score or lack of amenorrhea. Our sample consists of patients with moderate depression symptoms, without clinical diagnosis of MDD or drugs used in depression treatment. Proceeding from the above-mentioned assumptions the obtained results may be consider as specific for anorexia.

None of the perinatal stressful factors analyzed were found to co-occur significantly higher with the analyzed genotype polymorphisms in the patient group, not in accordance with the Connan neurodevelopmental theory of AN (Connan et al. 2003). This result may be due to the underestimation of the scale of perinatal problems arising from obtaining information from parents who do not always have medical documentation.

The development of AN is also considered to be a consequence of SLE during childhood and adolescence. Particularly important risk factors for eating disorders include such events as school problems, separation from family, death of a close family member and family conflicts (Berge et al. 2012). However, no correlation between SNPs of the NR3C1 gene and long-acting SLE were found in the present study. A potential reason for the lack of correlation may be either still too small group of respondents as well as the underestimation of long-acting SLE in our anorexia patients. In a previous

study, approximately 64% of AN patients experienced a traumatic event in life (Favaro et al. 2010), whereas we observed SLE in 51% of our study sample. Such differences may be due to a combination of both a cultural stigma and perfectionism in AN Polish patients, who are not eager to reveal family conflicts or violence (Bloks et al. 2004). Perhaps this is also why such a small number of patients completed the LEC. Regardless, it appear that the information obtained from LEC is reliable, as the average number of SLE related by our patients was 4.2 (SD±5.85), whereas it was reported to be 2.7 in adolescent twins (Chen et al. 2012).

The main limitation of the present study is the lack of information on SLE in the control group. However, the cohort study conducted by Sali et al. (2013) indicated that some life events occur in a healthy control group with similar frequency as in a group of patients, e.g., death of a close family member, loneliness or lack of understanding (Sali et al. 2013). This finding may indirectly indicate that there is no difference in the prevalence of SLE in healthy and AN patients, therefore resulting in a lack of a significant correlation between SLE and genetic variants. However, we should remember that the pattern of the frequency and severity of stress factors (first love, moving out of the house or having a baby) change over time. Therefore, additional research is necessary for further conclusions.

CONCLUSIONS

Our results confirm that the NR3C1 gene is associated with AN and indicate that a genetically programmed sensitivity to the stress axis, regardless of the type of stressful triggering factor, is important in AN development.

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