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Association Study Between the Triallelic Polymorphism of SLC6A4 Gene and Eating Disorders

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Abstract: The serotonin transporter is encoded by the SLC6A4 gene and has been an interesting candidate for anorexia nervosa (AN) and bulimia nervosa (BN). The present study analyzed the association between the triallelic model of the SLC6A4 gene and eating disorders in Mexican population. Materials and Methods: The 5-HTTLPR/rs25531 polymorphism was analyzed in 458 eating disorder patients and 337 control subjects. Genotype and allele analyses were examined in 206 BN and 79 AN patients and compared with the control group. Furthermore, genotype and allele analyses were performed on AN-Spectrum (AN-R, AN-BP and AN-EDNOS) and BN-Spectrum (BN-P, BN-NP and BN-EDNOS) groups and compared with the control group. Results: Case-control analysis showed that BN patients had an increased frequency of the S/L_G alleles compared to controls ($\chi2=6.9$, df=1, p=0.0088). However, no association was found between AN and the 5-HTTLPR/rs25531 polymorphism ($\chi2=3.3$, df=1, p=0.0654). Also, an association was observed in genotype distribution when comparing AN-spectrum and control groups ($\chi2=10.1$, df=2, p=0.0069); however, analysis of allele frequencies did not show differences after Bonferroni correction ($\chi2=5.6$, df=1, p=0.0177). Finally, analysis of BN-Spectrum showed a high frequency of S/L_G alleles compared to control group ($\chi2=7.3$, df=1, p=0.007). Conclusion: The low activity alleles of the 5- HTTLPR/rs25531 polymorphism of the SLC6A4 gene may play a significant role in the etiology of BN subtypes in Mexican population.

Keywords: Anorexia Nervosa, Bulimia Nervosa, Serotonin Transporter, 5-HTTLPR, rs25531

1. Introduction

Studies in animal models and humans indicate that manipulations of the serotonin system result in changes in eating behavior. The serotonin transporter plays an important physiological function in terminating the synaptic action of serotonin after the neurotransmitter release. The serotonin transporter is encoded by the SLC6A4 gene and has been an interesting candidate for anorexia nervosa (AN) and bulimia nervosa (BN). Several polymorphic variants that affect the expression or function have been identified in the SLC6A4 promoter region. One of them, 5-HTTLPR (serotonin transporter linked polymorphic region) is characterized by the insertion/deletion of 44-base pair that defines a biallelic system formed by S (Short) and L (Large) variants [1]. The S allele is associated with a reduced expression of 5-HTT mRNA and therefore in turn it leads to reduced 5-HT uptake from the synaptic cleft. Another variant, a single nucleotide polymorphism (SNP) rs25531, located in the sixth nucleotide within the first of two 20-23 bp repeats in the L allele of the ins/del polymorphism involves a substitution of G to A and designated as L_A and L_G, defines, together with the S allele, a triallelic system (5-HTTLPR/rs25531). Interestingly, the L_G variant contains an AP2 binding site that reduces its expression levels, showing a similar activity to the S allele when compared with the L_A variant, which has a high

expression level [2].

Five meta-analysis of studies analyzing the 5-HTTLPR polymorphism in ED have been performed. Four studies found evidence to support an association between the S allele and AN [3-6]. The five meta-analysis reported no significant association between 5-HTTLPR polymorphism and BN [7]. It has been suggested that the analysis of a promoter variant such as a triallelic system should provide additional information findings about the role of SLC6A4 variants in mental disorder [8]. Thus, the hypothesis of the present study is that the S/L_G variant of the 5-HTTLPR/rs25531 polymorphism is associated with eating disorders (ED) patients.

2. Materials and Methods

2.1. Participants

The study was carried out with patients consecutively attending the Clinic of Eating Disorders in the National Institute of Psychiatry Ramon de la Fuente Muñiz, in Mexico City. The sample was recruited through a project related to the genetics of anorexia nervosa and bulimia nervosa. A total of 458 patients (428 females and 30 males) were diagnosed using the Structured Clinical Interview for Mental Disorders v.2.0 (SCID-I) [9]. The sample consisted of 38 subjects (8.3%) fulfilling the DSM-IV-TR criteria for anorexia nervosa, restricting subtype (AN-R); 41 subjects (9%) fulfilling the criteria for anorexia nervosa, binge-eating/purging subtype (AN-BP); 187 (40.8%) for bulimia nervosa, purging subtype (BN-P); 19 (4.1%) for bulimia nervosa, non-purging subtype (BN-NP); 32 (7%) for AN-spectrum eating disorders not otherwise specified subtype (AN-EDNOS); and 141 (30.8%) for BN-spectrum EDNOS. The mean age of the sample was 20.7 years (SD 6.7) and the median age of onset was 13 years (SD 2.3).

The control group comprised 337 unrelated healthy subjects (170 females and 167 males) with no current or past psychiatric history screened using the Spanish version of the Diagnostic Interview Schedule (DIS), and all individuals had passed the age of risk for ED. The mean age of the control group was 40.1±12.5 years and BMI was 22.3±2.6 kg/m^2. All participants were Mexican Mestizos with a family background of three generations born in Mexico. All the participants provided written informed consent before the study according to the protocol approved by the Ethics Committee of the National Institute of Psychiatry Ramon de la Fuente Muñiz.

2.2. Genotyping

Genomic DNA was extracted from peripheral blood by a standard procedure. The analysis of the 5HTTLPR/rs25531 polymorphism was performed in two steps. The 5-HTTLPR was genotyped using the primers and the conditions previously reported [1]. PCR products were resolved on 1.5% high-melt agarose gels and visualized under UV illumination after ethidium bromide staining. Allele sizes were determined by comparison with a 50-bp DNA ladder.

SNP rs25531 was subsequently analyzed with a TaqMan SNP Genotyping Assay–by- Design using the forward primer 5′-ACCCCTCGCGGCATC-3′ and the reverse primer 5′-ATGCTGGAAGGGCTGCA-3′. The fluorogenic probes were 5′-VIC- CCCCCTGCACCCCCaGCA-MGB-NFQ-3′ and 5′-FAM-CCCTGCACCCCCgGCA- MGB-NFQ3′ [2]. The 5 µl reaction volume contained 10 ng of genomic DNA, 2.5 µl of TaqMan Master Mix (ABI) and 0.125 µl of 40X Assay mix in accordance with the manufacturer's recommendations (Applied Biosystems Inc). The amplification was performed in 96-well plates using the TaqMan Universal Thermal Cycling Protocol. Fluorescence intensity was measured with the 7500 real time PCR system using the SDS software v.2.1 (Applied Biosystems). Genotyping was performed blind to sample identity.

2.3. Statistical Analysis

The Hardy-Weinberg equilibrium was tested with the HWE software. Genotype and allele frequencies of patients and controls were analyzed with $\chi 2$ statistics, using the Tadpole Package, written by Caradoc-Davies, Elsevier-Biosoft, version R2. The results were corrected for multiple testing with the Bonferroni method, considering four comparison groups (significant p value< 0.0125). The power analysis was performed using the program QUANTO V.1.2 [10] showing in the sample a power of 0.9 to detect a two-fold increased risk, assuming an additive genetic model, a risk allele frequency of 0.57, an α level of 0.05 and a control-case ratio of 1:1.

3. Results

Analysis of genotype frequencies of 5-HTTLPR/rs25531 polymorphism was according to Hardy-Weinberg equilibrium for cases and controls (p>0.05).

Table 1 shows genotype and allele frequencies of the triallelic 5-HTTLPR/rs25531 polymorphism. There was an association in allele and genotype distribution between BN and control group ($\chi 2$=8.9, df=2, p=0.0118; $\chi 2$=6.9, df=1, p=0.0088). There were not differences in genotype or allele frequencies between AN and control subjects ($\chi 2$=4.7, df=2, p=0.0933; $\chi 2$=3.3, df=1, p=0.0654) nor in the comparison of AN and BN groups ($\chi 2$=0.09, df=2, p=0.9512; $\chi 2$=0.0005, df=1, p=0.9798) (Table 1).

In addition, the analysis of the triallelic 5-HTTLPR/rs25531 polymorphism in BN-spectrum (BN-P, BN-NP and EDNOS-BN) and control group, a statistical association was found ($\chi 2$=11.1, df=2, p=0.0043; $\chi 2$=7.3, df=1, p=0.007). In addition, there was an association in the comparison of AN-spectrum (AN-R, AN-BP, EDNOS-AN) and control groups ($\chi 2$=10.1, df=2, p=0.0069; $\chi 2$=5.6, df=1, p=0.0177); however, the analysis of allele frequencies did not show differences after Bonferroni correction (Table 1).

Table 1. *Genotype and allele frequencies of the 5-HTTLPR/rs25531 polymorphism.*

Groups	Genotype			Allele	
	SS, SL_G, L_GL_G	**SL_A, L_AL_G**	**L_AL_A**	**S, L_G**	**L_A**
AN (n=79)	31 (0.39)	41 (0.52)	7 (0.09)	103 (0.65)	55 (0.35)
BN (n=206)	83 (0.40)	103 (0.50)	20 (0.10)	269 (0.65)	143 (0.35)
AN-Spectrum (n=111)	43 (0.39)	61 (0.55)	7 (0.06)	147 (0.66)	75 (0.34)
BN-Spectrum (n=347)	135 (0.39)	177 (0.51)	35 (0.10)	447 (0.64)	247 (0.36)
Controls (n=337)	113 (0.30)	160 (0.45)	64 (0.03)	386 (0.57)	288 (0.43)

4. Discussion

Conflicting findings have been reported regarding the association between ED and SLC6A4 gene polymorphisms. Four meta-analyses in case-control association studies using the biallelic 5-HTTLPR model demonstrated a genetic susceptibility of the S allele to ED, especially for AN [3-5]. In addition, a meta-analysis study using a dominant and additive model showed a non-significant association between the 5-HTTLPR polymorphism and BN [7].

SLC6A4 gene variants have shown significant variation worldwide; therefore, findings cannot be extrapolated a priori across populations [11]. There are ethnic differences in the allele distributions of triallelic 5-HTTLPR/rs25531 variants; Murphy et al. showed the frequency of low-activity alleles (S/L_G) in Caucasians is 22%, in American-Indians 43% and in Asians 60% [8]. The present study showed that the Mexican Mestizos have a frequency of 57%, a similar frequency to Asian population.

Previously, a 48% frequency of the L allele was reported in a sample of 136 control subjects [12]. In the present study, using a triallelic model, the analysis showed that 6.6% of the L alleles were carriers of the G variant (L_G), which allowed us to determine that the allele frequency of the high-activity variant (L_A) was 43% in a sample of 337 Mexican control subjects.

In the present study, a positive association between the 5-HTTLPR/rs25531 polymorphism and BN patients was observed. Interestingly, the results found a high frequency of S/L_G alleles in BN patients. Di Bella et al. reported an association between the S allele and BN; however, a deviation from the Hardy-Weinberg equilibrium was detected [13]. Two studies found an association between the S allele and AN, whereas others did not confirm it [14-18]. In contrast, a higher frequency of the L allele in BN females was reported in a previous study [19]. In addition, an analysis of the triallelic model showed an association between the L_A allele and the inhibited/compulsive subgroup, which was composed of a higher proportion of AN patients [20].

Also, the AN group showed higher S/L_G allele frequencies compared with the control subjects, but these differences were not statistically significant. However, the analysis of AN-Spectrum demonstrated an association in the genotype distribution compared with the control group. This result should be considered as preliminary due to the small sample size of this subgroup.

The positive association observed in the ED sample may be related to the phenotypic characteristics of the disorders. AN, BN, and EDNOS patients share clinical features. The sample showed a clear, high frequency of S/L_G alleles in anorexic and bulimic patients; therefore, it is possible that the low-activity alleles may be related to a sub-phenotype among ED patients. Interestingly, it was reported that an increase in depression symptoms over time was strongly associated with an increase in bulimic symptoms in patient carriers of the SS genotype compared with carriers of at least one L allele [21]. In addition, it was reported that BN patients who were carriers of the S allele and had a history of abuse showed elevated psychopathology [22-24]. In addition, Monteleone et al. reported higher harm avoidance scores in BN patients who were carriers of SS and SL genotypes [19].

The findings showed a higher frequency of low-activity variants (S/Lg) in bulimic disorders, being consistent with other studies indicating reduced serotonin activity in syndromes characterized by affective and behavioral dysregulation [21, 25].

Previously, a higher frequency of the rare Ala56 variant of the SLC6A4 gene in ED Mexican patients has been reported [26]. Therefore, it has been important to identify other common and rare variants located in the SLC6A4 gene that might be acting in linkage disequilibrium with S/Lg and Ala56 alleles in the risk to develop an ED. Furthermore, it seems that a genetic analysis of clinical and behavioral traits is required to try to understand the role of SLC6A4 gene variants among ED patients.

5. Conclusion

The findings from this study support the hypothesis that the low-activity variants of SLC6A4 gene may be associated with bulimia nervosa. Therefore, future studies in a larger sample size should include multiple polymorphisms located in the SLC6A4 gene to confirm the results.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contributions

BC designed the study and wrote the manuscript. SH and AA carried out the genotyping. SH contributes in the statistical analysis. LG, AC, GF and DL carried out the clinical evaluation of the patients. All authors read and approved the final version.

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