

A study of blood serotonin and serotonin transporter promoter variant (5-HTTLPR) polymorphism in Egyptian autistic children

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Abstract

Background: Autism spectrum disorder (ASD) is a complex, heterogeneous neurodevelopmental disorder with onset during early childhood. Most studies have reported an elevation in platelet serotonin in persons with autism. The serotonin (5-hydroxytryptamine; 5-HT) transporter in the brain uptakes 5-HT from extracellular spaces. It is also present in platelets, where it takes up 5-HT from plasma. Polymorphisms in serotonin transporter gene (SLC6A4) were frequently studied in many neuropsychiatric disorders.

Materials and Methods: We have measured the plasma 5-HT levels in 20 autistic male children and 20 control male children by the enzyme-linked immunosorbent assay (ELISA) method. In addition, the SLC6A4 promoter region (5-HTTLPR) insertion/deletion (I/D) polymorphism was studied, using whole genomic DNA.

Results: Plasma serotonin was significantly low in autistic children compared to control ($P = 0.001$), although correlation to severity of autism was not significant. The frequency of short (S) allele in autism cases was 10% and in the control group it was absent.

Conclusion: Our study demonstrated an increased prevalence of 5-HTTLPR S allele in autism subjects. Significantly decreased plasma serotonin was detected in autism subjects, with no significant relationship between 5-HTTLPR genotype and plasma 5-HT being evident.

Key Words: Gene promoter, platelet, reuptake, serotonin, synapses, transport

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INTRODUCTION

The incidence of autism is increasing worldwide, while the underlying pathophysiological mechanisms remain

virtually uncharacterized. The rate of prevalence is estimated to be 1 in 110 in the US, and there is a 1.1% prevalence rate in the UK with similar incidences throughout the world (Brugha, *et al.*, 2012).^[1]

There are classes of different pharmacological agents that are found to be effective in improving behavioral symptoms of autism spectrum disorder (ASD), serotonin reuptake inhibitors being one of them (Hubbard, *et al.*, 2012).^[2] Therefore, serotonin (5-hydroxytryptamine; 5-HT), an neurotransmitter found throughout the brain, has been a potent interest in studies on autism.

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There are many reports of elevated platelet 5-HT in autism, and of its dysfunctional signaling as a causal mechanism for the disorder (Mulder, *et al.*, 2004; Yubero-Lahoz, *et al.*, 2013).^[3,4] A recent review study showed the similarity of platelet 5-HT to neuronal serotonin and thus the use of platelets' 5-HT activity as a peripheral marker for its central activity (Yubero-Lahoz *et al.*, 2013).^[4] A disturbance of serotonin in autistic children is thought to be linked to carbohydrate-rich diets (Vered, *et al.*, 2003)^[5], as a significant elevation in 5-HT levels in autistic children was noted shortly after the meal with a significant decreased level in a steeper form thereafter that would be attributed to increased serotonin reuptake from the blood by either platelets or neurons in the brain.

Platelet serotonin, also known as platelet-poor plasma (PPP) serotonin, showed variable results in autism and altered handling of serotonin was suggested. The level of PPP 5-HT in autism was inversely related to the severity of autism (Spivak, *et al.*, 2004; Anderson, *et al.*, 2012).^[6,7] The same trend was also found in mothers of autistic children during pregnancy, and an in-utero role for serotonin in fetal susceptibility for developing autism was highlighted (McBride, *et al.*, 1989).^[8] A recent study suggested the dependency of the raphe-prefrontal network (mainly the medial prefrontal cortex) on the 5-HT transporter during early development of the brain, and this area is responsible for many neurodevelopmental disorders (Witteveen *et al.*, 2013).^[9]

The serotonin transporter gene (SLC6A4) has been studied in the context of ASDM (Makkonen, *et al.*, 2008; Buznikov, *et al.* 2001).^[10,11] SLC6A4 is a transporter protein that transports serotonin from the synaptic cleft to the presynaptic neuron and thus terminates the action of serotonin. SLC6A4 gene transcription is influenced by polymorphisms, which were extensively studied in many neuropsychiatric disorders, with two commonly studied polymorphisms in its promoter region (Bonnin and Levitt, 2011; Lesurtel *et al.*, 2006; Anderson *et al.*, 2009).^[12-14] Serotonin-transporter-linked polymorphic region (5-HTTLPR) was studied for association with ASD in many populations, with controversial results depending on ethnic diversity, methods of genetic analysis, and symptoms of ASD (Gordon, *et al.*, 1993; McDougle, *et al.*, 1996; Heils, *et al.*, 1996; Lesch, *et al.*, 1994).^[15-19] 5-HTTLPR polymorphism due to a 44-base pair (bp) insertion/deletion (I/D) was linked to different protein expression and thus activity of corresponding protein. The long (L) allele of 5-HTTLPR is thought to be linked to higher 5-HT transporter expression (Yirmiya, *et al.*, 2001; Kim, *et al.*, 2002)^[20,21] and thus lower blood levels of 5-HT.

The aim of this study is to report the difference in PPP 5-HT levels and determine 5-HTTLPR I/D polymorphism in a sample of autistic children and age-matched controls.

MATERIALS AND METHODS

Patients and participants

Our study included 20 autistic male children aged 7.4 ± 2.6 years and 20 control children aged 9 ± 1.6 years. Their age ranged 5-15 years (mean age 6.6 ± 4.4 years).

The diagnosis of autism was confirmed by the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) and the Childhood Autism Rating Scale (CARS) (Schopler *et al.*, 1999).^[22] The cases were invited to participate in the study and the Research Ethics Committee of the National Research Centre approved all procedures for blood donation and research. The caregivers' consent was obtained for all the studied cases.

Molecular analysis

Whole genomic DNA was isolated from the blood of both the autistic children and the control group. The DNA purity and concentration were measured by NanoDrop (ND)-1000 (Wilmington, DE 19810 USA). All used samples showed a concentration of 200 ng/ μ l and their purity was determined by the 260/280 ratio. The 5-HTTLPR I/D genotype was determined via polymerase chain reaction (PCR), following a previously described protocol (McCauley, *et al.*, 2004).^[23] The forward primer 50 - GGCGTTGCCGCTCTGAATGC - 30 and the reverse primer 50- GAGGGACTGAGCTGGACAACCAC - 30 were used. Cycling conditions included an initial denaturation at 95°C for 10 min, followed by 50 cycles of 94°C for 15 s, optimal annealing temperature (T_{opt}°C) for 30 s, 72°C for 15 s, and a final extension at 72°C for 10 min. The PCR products were run on 3% agarose gel stained with ethidium bromide. The L and S alleles corresponded to 523- and 484-bp fragments, respectively, [Figure 1].

Heterozygous samples showed both the alleles. Two investigators scored the allele sizes independently. Any inconsistency was reviewed and procedures were repeated if necessary. Genotyping was conducted blind to both the cases and the control samples without access to the clinical information.

Determination of PPP 5-HT

The serotonin assay was done using Serotonin ELISA kit (DIASource ImmunoAssays S.A. 8, Rue de l'Industrie, B-1400 Nivelles, Belgium), using the competitive ELISA kit (the microtiter plate format).

All statistical analysis was done using the SPSS version 14 software package (IBM SPSS Statistics, USA) and graph prism program. The Chi-square test was used to evaluate the difference in gene alleles between the studied groups, and P values of 0.05 were considered statistically significant. Multinomial logistic regression was used to study interacting factors.

RESULTS

The autistic patients and the control group showed matched ages with no significant difference ($P = 0.3$). They are all male children. The PCR results for autistic patients showed the allele frequencies to be 10% for the S allele and 90% for the L allele [Table 1]. Four autistic cases had the L/S 5-HTTLPR genotype, and the distribution of L/L, L/S, and S/S genotypes among patients were 80%, 20%, and 0% (with 10% and 90% allele frequency for S and L alleles, respectively). In the control group, the S allele was not detected.

The Chi-square test of allele distribution among autistic patients and controls shows a significant association of the S allele with autism ($P = 0.035$). No effect of age was noted. As all cases are males, the effect of sex on polymorphism could not be studied.

Blood serotonin

Measured serotonin levels in autism cases ranged 43-159 ng/ml (mean $75 \pm$ SD 43), and in control 139-171 ng/ml (mean $157 \pm$ SD 10) [Figure 2]. There is a significant decrease in the serotonin level in autistic children compared to control, as seen by using the Mann—Whitney test ($P = 0.001$) [Table 1].

The present study included 11 patients with severe autism (CARS 39-60) and 9 patients with mild

to moderate autism (CARS 30-38). No significant difference in blood serotonin levels between the moderate group (mean $73.11 \pm$ SD 40.87) and the severe autistic features group (mean $85.54 \pm$ SD 50.18) was found.

We could not detect a significant difference in blood serotonin with relation to different 5-HTTLPR genotyping among autism cases ($P = 0.14$).

DISCUSSION

Our study shows a significant decreased PPP 5-HT level in autistic children compared to control.

Measurement of the PPP levels of 5-HT appears to provide the best available index of *in vivo* exposure of the platelet to 5-HT (Anderson, *et al.*, 2012).^[7] An interesting study measuring 5-HT axons that were immunoreactive to a serotonin transporter (5-HTT) antibody in a number of postmortem brains from autistic patients, showed that the number of serotonin axons was increased in them (Azmitia, *et al.*, 2011).^[24] Another study found similarities in brain and plasma 5-HT levels in response to dietary factors (Kot, *et al.*, 2012).^[25]

Table 1: 5- HTTLPR genotype and allele frequencies among autistic and control children

	Autistic children (%)	Control children (%)
5-HTTLPR genotype		
5-HTTLPR L/S	4/20 (20)	0
5-HTTLPR L/L	16/20 (80)	20 (100)
5-HTTLPR allele frequency		
Short allele	4/40 (10)	0
Long allele	36/40 (90)	40/40 (100)
Blood serotonin level		
Mean \pm SD	75 \pm 43	157 \pm 10

5-HTTLPR: Serotonin-transporter-linked polymorphic region, SD: Standard deviation

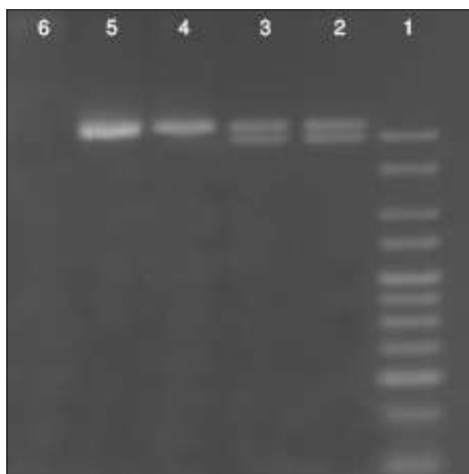


Figure 1: Agarose gel 3% showing the PCR products for 5-HTTLPR I/D genotype of 4 autism cases (lane 2&3 are L/S heterozygotes, lane 4&5 are L/L homozygotes, while lane 6 is a negative control)

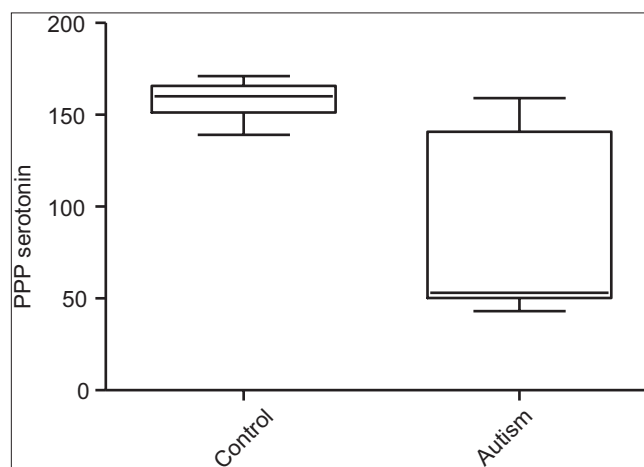


Figure 2: Plasma serotonin between autism and control groups ($P = 0.001$)

Our results of decreased PPP 5-HT represented an indicator of decreased central 5-HT due to increased neuronal uptake. A study done on pigs found an interrelated value between the brain (hippocampus) and the platelet serotonin. Both values were related to some exploratory tests in pigs (Ursinus, *et al.*, 2013).^[26] It is suspected that the relationship would be more complicated in autism, with serotonin being one of the implicated central neurotransmitters and its metabolism being a predisposing mechanism.

Serotonin neuronal cellular effects were studied recently (Mercado *et al.*, 2013)^[27] using microarray technology, the main biological pathways affected being cytoskeletal remodeling, protein signaling, and apoptosis. In the present work, autism cases showed significantly increased 5-HTTLPR S allele compared to controls ($P = 0.035$). The findings of the present study are consistent with those of a previous study by Anderson *et al.* 2012.^[7] However, they included first-degree relatives, e.g., mothers, fathers, and sibling as control groups, which is considered one of the limitations of that study, while the present study enrolled unrelated, normal, healthy children.

Our study is the first work on Egyptian autistic children to study changes in blood serotonin and the 5-HTTLPR L/S polymorphism. A previous study carried out on South African autistic patients showed a significantly higher prevalence of 5-HTTLPR S allele among autistic patients, with a frequency of 33%, and the absence of the same in the control group (Arieff, *et al.*, 2010),^[28] which is similar to our results. The discrepancy between our genotyping results and other studies could be attributed to the various ancestral populations that were proved regarding 5-HTTLPR polymorphism (Esau, *et al.*, 2008).^[29]

The 5-HTTLPR L/S genotyping in the present study results is consistent with earlier reports on other populations such as the Indian population, as a significant preferential transmission of S allele from parents to the affected offspring was noted ($P = 0.006$), indicating an association of 5-HTTLPR with autism (Guhathakurta, *et al.*, 2006).^[30]

No difference is seen in serotonin levels in the autism cases with various HTTLPR genotyping. Serotonin levels could be influenced by other genetic markers than HTTLPR in SLC6A4, as this is supported by a recent study on autism in families that did not show any association of some social impairment tests with 5-HTTLPR polymorphism (Neves Mde, *et al.*, 2011),^[31] while another study found an association between SLC6A4 SNP rs16965628, located in intron one, and some behavioral problems (Lindholm Carlström

et al., 2012).^[32] It was also found that vitamin D (calcitriol) activates the transcription of the serotonin-synthesizing gene tryptophan hydroxylase 2 (TPH 2) in the brain (Patrick and Ames, 2014).^[33]

5-HTTLPR polymorphism was found to be associated with other psychiatric disturbances, e.g. childhood depression (Comasco, *et al.*, 2013),^[34] which would affect the symptoms of autism and the age of diagnosis. Fewer studies showed no main effect of 5-HTTLPR genotype and childhood depression (Tomoda, *et al.*, 2013).^[35]

The role of polymorphism in autism risk is thought to be influenced by environmental circumstances, e.g. social and parental rejection (Lindell, *et al.*, 2012).^[36]

To conclude, our study showed a significant correlation of the S allele of 5-HTTLPR with autism, and decreased serotonin in blood was detected among autistic children with no relationship between the studied polymorphism and serotonin levels.

This finding supports the role of 5-HTTLPR S allele as a risk factor for autism, although the correlation of genotyping with peripheral serotonin level would represent a complex biological process with many interacting genetic factors.

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