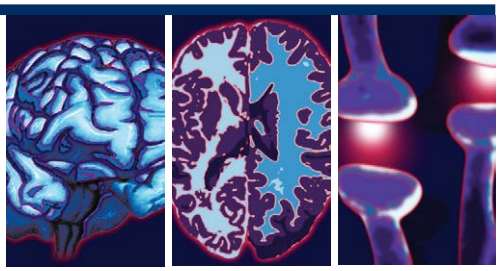
Research Article



Polymorphisms rs6313 and rs6314 in Serotonin Receptor Gene (*HTR2A*) and Serotonin Concentration in Autistic Children

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ABSTRACT

Objective

Genetic polymorphisms and mutations in candidate genes are considered important in the etiology of autism, and particular interest is focussed on the serotonin system. Here, we used SNP analysis in the serotonin receptor gene to identify differences between autistic and healthy control children.

Methods

Genetic association of rs6313 (T102C) and rs6314 (C1354T) polymorphisms in *HTR2A* gene with susceptibility to the development of autism in children were investigated using PCR-RFLP, and correlated serotonin levels in blood serum using ELISA method.

Results

We uniquely found an association between the presence of the *T* allele at the position rs6313 (OR=2.00, 95%CI: 1.23-3.26, p=0.005), and between the presence of the *C* allele at the position rs6314 (OR=2.24, 95% CI: 1.47-3.42, p=0.0002) of the serotonin receptor gene under conditions of a decreased ASD incidence. We also noted that *T* allele at the position rs6313, and *C* allele at the position rs6314 was 3 times more frequent in Control males than in ASD males. We found no statistical significant correlation between *HTR2A* SNPs and the blood serum level of serotonin between autistic and control children.

Conclusion

This study shows the involvement of *HTR2A*, rather than the concentration of serotonin, in the development of autism, and provides a rationale for future design of therapeutic modalities based on the serotonin system for childhood autism.

Keywords

HTR2A, Serotonin, Serotonin receptor, SNP, Polymorphism, Autism, ASD

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by abnormalities in social interactions, communication skills and restrictive or repetitive behaviors [1,2]. An important role in the etiology of autism is attributed to genetic factors and mutations in specific genes. The search for so-called candidate genes is ongoing, with the ultimate goal to become a marker

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of the disease and facilitate early diagnosis permitting to start treatment as soon as possible. Despite the fact that autism is considered as a multigenic disease, attention should be also paid to the role of single genes in the etiology of disease. Genes involved in the regulation of serotonin system are considered important risk factors in ASD [3,4] since Schain and Freedman [5] reported a characteristic high content of serotonin in the blood of autistic individuals. According to Kolevzon et al. [6] approximately one-third of autistic individuals are considered to have high serotonin levels in blood called as hyperserotonemia.

Serotonin (5-HT) is a monoamine neurotransmiter, synthesized from the essential amino acid tryptophane, and plays an important role in brain development, during the prenatal and postnatal period. It regulates emotional expression, social behaviour, and proliferation of immune system cells [3,7]. The effect of serotonin deficiency, the lack of its transporters, receptors or enzymes of the serotonin pathway are implicated in many diseases, including depression, numerous mood swings, emotional instability, schizophrenia and other neurological disorders, including autism [8-13]. Hadjikhani [14] hypothesized that increased serotonemia during pregnancy, including due to selective serotonin reuptake inhibitors intake, could be one of the causes of the raising prevalence in autism.

Serotonin regulates its function via 5-HT (HTR) receptor family members [3]. Many single-nucleotide polymorphisms (SNPs) have been described in the genes coding for the different types of serotonin receptors in humans [15]. In particular, studies have focused on the 5-HT2A receptor gene (*HTR2A*), as one of the most plausible functional candidate genes of ASD [10]. The human serotonin receptor *HTR2A* gene has been cloned and mapped to the chromosome 13q14–q21 region [16], it consists of 3 exons and 2 introns [9], and encodes a 471 amino acid mature protein (GenBank: AAH69356.1).

The genetic variants (SNPs) rs6313 (T102C, in exon 1), and rs6314 (C1354T, His452Tyr, in exon 3) within the serotonin receptor gene are associated with ASD [17,18], schizophrenia [19], depression [20], compulsive disorder (OCD) [21,22]. It has been also suggested that the serotonin 2A receptor gene (*HTR2A*) is associated with suicidal and/or impulsive aggressive behavior [23].

In the present study, we investigated association of *HTR2A* gene polymorphisms rs6313 (T102C) and rs6314 (C1354T) with autism spectrum disorder in affected children and correlated these levels with serotonin concentration in blood serum.

Materials and Methods

■ Control and patient characteristics

The autistic patients (ASD, ICD-F84) (90 male, 25 female, mean 6.9 years, range 5-12 years (ASD group) were recruited by specialists in the Center for Diagnosis, Treatment and Therapy of Autism at the Regional Children's Hospital in Olsztyn, Poland. The patient group was homogeneously selected, as described in earlier publications [24,25]. The autistic symptoms were assessed by means of the Childhood Autism Rating Scale (CARS), and according to Classification of Mental and Behavioral Disorders ICD-10 all patients had full-symptoms, nuclear form of Kanner autism (F84.0). Patients received Werthmann's hypoaller-genic diet and were not covered by a psychotropic drug therapy. F84.0 disease in children was identified on the basis of an interdisciplinary differential diagnosis: psychiatric examination excluding mental illness combined with studies evaluating cognitive parameters in the respondents; neurological examination, including EEG; evaluation of reflexes; speech therapy to evaluate the development of speech; passive and participatory observation lasting from 6 to 12 months; and finally analysis of the documentation: names of parents, the opinions of educational institutions, video recording. The IQ level was evaluated by a cognitive development tests, including the Leiter test and the Wechsler test (Leiter scale - standard IQ from 70 to 107; Wechsler – standard IQ from 90 to 104). In the ASD group, most children had IQ's of 70–104, and 9 children with IQ's of <70, which indicates mental retardation. Here we refer to ASD, as used in the DSM-V nomenclature, and the ICD-10 classification is widely used in medical centers. The included children were screened for history of neurological, psychiatric and developmental disorders and all were un-medicated (including no psychotropic medication) and in good general health at the time of participation. Each patient also had a basic neurological examination and an EEG was recorded. Exclusion criteria were the presence of known neurological disorders including fragile-X syndrome and tuberous sclerosis, congenital

metabolic disorders, chronic infectious diseases such as tuberculosis, acute infectious disease within the last 4 weeks, immunization within the last 8 weeks and immune-modulating medication in the previous four weeks.

In addition, a control group was included that comprised 176 healthy children with no history of behavioral disorders (105 females and 71 males with 5–17 years age-range and 7.9 mean age) recruited from the emergency department of the Regional Children's Hospital. All children in this study were Caucasian. Informed consent was obtained from all children's parents and the study was approved by the Local Bioethics Committee (No. 19/2016; 18/5/2016).

■ Polymorphisms in 5-*HTR2A* (rs6313, rs6314) gene in healthy and ASD children

DNA was isolated from peripheral blood using GeneJET™ Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Polymorphisms rs6313 and rs6314 were assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primer sequences examining these polymorphisms were previously described by Shimizu et al. [26] and together with the restriction enzyme listed in **Table 2**.

PCR amplification was conducted in a thermal cycler according to the following program: initial denaturation: 94°C for 3 min, denaturation: 94°C for 30s, attaching the starters at 61°C for both polymorphisms for 30s, synthesis: 72°C for 30s, final synthesis: 72°C for 5 min, number of cycles: 40, cooling: 4°C. The mixture in the volume of 25 µl consisted of DreamTaq™ Green Master Mix (Thermo Scientific, Waltham, USA), specific primers, the DNA matrix, and ultrapure water (Sigma-Aldrich, Saint Louis, USA). The yield and specificity of PCR products were evaluated by electrophoresis in 1.5% agarose gel (Promega, Fitchburg, USA) and staining with GelGreen Nucleic Acid Gel Stain (Biotium, Hayward, USA). Amplified fragments were digested with the appropriate restriction enzymes (Thermo Scientific, Waltham, USA) (**Table 2**) according to the manufacturer's instructions and visualized on a 2.5 % agarose gel. DNA sequencing of random chosen samples after amplification was used to confirm proper genotyping.

■ Serotonin concentration

The study included 39 patients (19 ASD children, and 20 healthy) from whom peripheral blood was collected. The selection of children was carefully made on the basis of age (6-9 years old), with no psychotropic medication use (**Table 1**). The analysis was immediately performed after blood collection in duplicate using Serotonin ELISA kit according to the manufacturer's instruction (LDN, Labor Diagnostika NORD, Germany). All steps of the ELISA were carried out at RT with gentle shaking (250 rpm) in microplate incubator (SkyLine ELMI Shaker DTS-4, Riga, Lithuania). Samples were acylated before analysis as follows: 25 µL of serum, standards or controls was mixed with 500 µL of acylation buffer and 25 µL of acylation reagent. The mixtures were incubated for 15 minutes at RT. Serotonin content was measured as follows: a standard curve in concentration 10.2–2500 ng/mL controls and serum samples were pipetted into serotonin microtiter strips. Then, 100 µl of the serotonin antiserum was added into all wells and incubation was carried out for 30 minutes. Plate was washed three times with Wash Buffer and 100 µl of the conjugate was added. After 15 minutes of incubation, 100 µL of substrate was pipetted. 15-minute incubation was repeated and 100 µL of stop solution was added. The absorbance was measured at a wavelength of λ= 450 nm using an ELISA reader (BiogenetAsys UVM 340, Cambridge, UK).

■ Statistical analysis

The genotype distribution among subjects was analyzed for Hardy-Weinberg equilibrium (HWE) using the chi-square test, and genotype

Table 1: Demographic characteristics.

	ASD group	Control group
SNP analysis		
Age, mean (range)	6.9 (5-12)	8.9 (5-17)
Male n (%)	90 (78.0)	71 (40.0)
Female n (%)	25 (22.0)	105 (60.0)
Serotonin concentration analysis		
Age, mean (range)	7.1 (6-9)	7.7 (6-9)
Male n (%)	15 (79.0)	13 (0.65)
Female n (%)	4 (21.0)	7 (0.35)

and SNP allele frequencies were compared between ASD patients and control groups by Fisher's test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression analysis and used to compare both allele frequencies in healthy controls and ASD patients. The risk of ASD development was estimated via wild-type genotype versus the wild/mutant and mutant-type genotypes. Serotonin concentration results have been presented as a mean ± SEM. The mean values in control and ASD groups were compared using ANOVA and Student's t-test. Statistical analysis was conducted on GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA), with ≤ 0.01 P-value considered statistically significant.

Results

■ Polymorphism of 5-*HTR2A* genes in healthy and autistic children

The observed genotype frequencies of rs6313 and rs6314 polymorphisms in the serotonin receptor gene (*HTR2A*) in 115 of healthy controls and 176 patients with ASD conformed to the Hardy-Weinberg equilibrium.

At the rs6313 polymorphic site, the 3 genotypes TT, TC and CC were identified with 0.57 *C* allele frequencies in the entire research population. Of the total 291 participants, 85 had genotype CC, 155 had TC and 51 had TT.

At the rs6314 polymorphic site the frequency of alleles *C* and *T* were determined in healthy children and in those diagnosed with ASD in our study population. Three genotypes (CC, TC and TT) were identified in the whole study population, with allele *C* frequency of 0.80. Of the total 291 participants, 190 had genotype CC, 85 had TC and 16 had TT.

Tables 3 and 4 show the genotype distributions, allele frequencies and associations between genotype and ASD incidence at the rs6313 and rs6314 polymorphic site in healthy children (Control) and patients with ASD, and association with gender. These results suggested an association between the presence of the *T* allele at the position rs6313 (OR=2.00, 95% CI: 1.23-3.26, p=0.005), and between the presence

Table 2: Primers for serotonin SNPs rs6313 and rs6314 and PCR-RFLP conditions.

SNP	Primer sequence	Restriction enzyme	PCR/RFLP products(bp)
Rs6313	6313L:5′-TCTGCTACAAGTTCTGGCTT-3′ 6313:5′-CTGCAGCTTTTTCTCTAGGG-3′	**Fast Digest MspI (HpaII)**	**CC: 126, 216 bp TT: 342 bp TC: 126, 216, 342 bp PPCR: 342 bp**
Rs6314	6314L:5′-AGATGCCAAGACAACAGATAATGAC-3′ 6314R: 5′- CTCACCTTTTCATTCACTCCG -3′	**Fast Digest BsmI (Mva1269I)**	**CC: 52, 53 bp TT: 105 bp TC: 52, 53, 105 bp PPCR: 105 bp**

Table 3: Genotype and allele frequencies of serotonin receptor SNP rs6313 polymorphism in studied groups and autism association.

Research group	% of genotypes			Frequency of alleles	
	TT	TC	CC	T	C
Control Group *(n=176)*	21	53	26	0.47	0.53
ASD Group *(n=115)*	12	54	34	0.39	0.61
ASD + Control Group (n=291)	18	53	29	0.43	0.57
Male (ASD + Control) *(n=161)*	20	51	29	0.46	0.54
Female (ASD + Control) *(n=130)*	14	56	30	0.42	0.58
Male Control *(n=71)*	31	44	25	0.53	0.47
Male ASD *(n=90)*	12	57	31	0.41	0.59
Female Control *(n=105)*	14	59	27	0.44	0.56
Female ASD *(n=25)*	12	44	44	0.34	0.66
	OR (95% CI)			**p-value**	
	TT *vs.* TC+CC				
Control *vs.* ASD group	**2.00 (1.23-3.26)**			**0.005**	
Male *vs.* Female (ASD + Control)	1.59 (1.00-2.52)			0.05	
Male *vs.* Female (Control)	2.58 (1.49-4.49)			0.0008	
Control Male *vs.* ASD Male	**3.19 (1.76-5.80)**			**0.0001**	

CI: Confidence Interval; OR: Odds Ratio.

of the *C* allele at the position rs6314 (OR=2.24, 95% CI: 1.47-3.42, p=0.0002) of the serotonin receptor gene under conditions of a decreased ASD incidence. We also noted that *T* allele at the position rs6313, and *C* allele at the position rs6314 were 3 times more frequent in Control Males than in ASD Males (**Tables 3 and 4**).

■ Serotonin concentration in serum

Average serotonin concentration in ASD group was 249.8 ng/ml (120.0-380.0 ng/ml; SDE=17.6), and in Control group 239.6 ng/ml (166.0-374 ng/ml; SDE=12.9) with no statistical significant difference. There were also no statistical significant differences according to individual serotonin receptor SNPs (rs6313, rs6314) and serotonin concentration in serum of autistic groups (**Table 5**).

Discussion

Causes of autism are widely described to be multifactorial and include both genetic and environmental factors [27,28], and it is important to search for new methods of diagnosis and prevention. Single nucleotide polymorphism analysis in autism can identify risk factors for this disease by their presence as genomic markers. As suggested [29] the serotonin system is important for treatment in a ASD children. Serotonergic system receptors (5-HTR) are responsible for the proper functioning of the serotoninergic system, which plays the role of neurotransmitter during brain development. Also hyperserotonemia in autistic children has been characterized as reduction in 5-HT2A receptor binding activity [29,30], and reduction in the number of serotonin receptors [31].

In this study we used SNP analysis to identify the differences between ASD and healthy controls in the distribution of rs6313 and 6314 polymorphism genotypes in the serotonin receptor *HTR2A* gene (**Tables 3 and 4**). The inspiration for the present study was reports in literature of different serotonin concentrations in children with autism. Elevated whole blood serotonin, or hyperserotonemia, was the first biomarker identified in autism spectrum disorder (ASD) and was found to be present in about 25-50% of affected children. Some studies suggested a relationship between autism and serotonin, and hyperseritonemia was suggested as a biomarker in ASD [32].

We showed that the frequency of alleles in autistic children for rs6313 (C102T) polymorphism (0.39 for T) was lower than data presented by Guhathakurta et al. [17] with T allele frequency–0.47 (genotypes: TT–0.22, TC– 0.50, CC–0.28), and Cho et al. [10]–0.51-0.55 for T allele (genotypes: CC–0.21, CT–0.47-0.53, TT–0.26-0.31). Our results uniquely suggest an association between the presence of the *T* allele at position rs6313 (OR=2.00, 95% CI: 1.23-3.26, p=0.005), of the *HTR2A* gene and a decreased ASD incidence. We also noted that allele *C* was three times more frequent in ASD Males than in Control Males (**Table 3).** Low *n* of ASD Females excluded the group from OR correlation.

In the research literature it was shown that 102 T/C (rs6313) polymorphism regulates the transcriptional efficiency of the gene [33], and reduces expression and function of the 5-HT2A receptor in autistic patients [34]. Studies have shown that the presence of the *C* allele in rs6313 polymorphic site is associated with lower mRNA and protein expression compared to the *T* allele [35]. Gong et al. [36] investigated the relationship between disorders in communication and social interaction with the level of serotonin in the brain in people with autism in correlation to SNP T102C in the *HTR2A* gene. It has been shown that autistic children had a higher frequency of the *C* allele, which is associated with lower activity of the 5-HT2A receptor. However, experiments of Cho et al. [10] and Guhathakurta et al. [17] on the *HTR2A* gene did not show a significant association between SNP rs6313 and the development of autism.

In our study, the presence of the *C* allele in rs6314 polymorphic site (C1354T) was decreased (0.73 for C allele) in ASD children compared to the value of 0.93 for the *C* allele as noted before [17]. The presence of the *C* allele at position rs6314 (OR=2.24, 95% CI: 1.47-3.42, $p \leq 0.0002$), and in Control Males (OR=3.38, 95% CI: 1.85-6.17, p=0.0001) of the *HTR2A* gene was associated with a decreased ASD incidence (**Table 4**). Low *n* of ASD females excluded the group from OR correlation.

Although higher serotonin levels have been demonstrated in children with autism (Table 5), we did not find this difference. In groups, ASD and control, the results showed high disproportions in serotonin concentration (120.0-380.0 ng/ml, 166.0-374.0 ng/ml, respectively), and mean value was no statistically significant (249.9 ng/ml and 239.6 ng/ml, respectively).

In reference to the literature elevated blood

Table 4: Genotype and allele frequencies of serotonin receptor SNP rs6314 polymorphism in studied groups and autism association.

Research group	% of genotypes			Frequency of alleles	
	TT	TC	CC	T	C
Control Group *(n=176)*	2	27	71	0.15	0.85
ASD Group *(n=115)*	13	37	65	0.27	0.73
ASD + Control Group (n=291)	6	29	65	0.20	0.80
Male (ASD + Control) *(n=161)*	7	31	62	0.22	0.78
Female (ASD + Control) *(n=130)*	4	27	69	0.17	0.83
Male Control *(n=71)*	0	25	75	0.13	0.87
Male ASD *(n=90)*	12	36	52	0.30	0.70
Female Control *(n=105)*	3	29	69	0.17	0.83
Female ASD *(n=25)*	8	20	72	0.18	0.82
	OR (95% CI)			**p-value**	
	CC *vs.* TC+TT				
Control *vs.* ASD group	**2.24 (1.47-3.42)**			**0.0002**	
Male *vs.* Female (ASD + Control)	0.69 (0.45-1.06)			0.09	
Male *vs.* Female (Control)	1.47 (0.79-2.73)			0.22	
Control Male *vs.* ASD Male	**3.38 (1.85-6.17)**			**0.0001**	

CI: Confidence Interval; OR: Odds Ratio.

Table 5: Correlation between serotonin receptor polymorphisms (rs6313 and rs6314) and serotonin concentration (ng/ml) in autistic and control group.

Serotonin receptor gene polymorphism	No of patients	Concentration of serotonin mean (range) (ng/ml)	SDE	P-value
Autism				
rs6313				
TC	6	252.2 (178.0-308.0)	19.97	0.53
CC	10	235.1 (120.0-380.0)	30.36	
TT	3	294.0 (245.0-323.0)	24.64	
rs6314				
TC	7	261.4 (120.0-380.0)	38.32	0.63
CC	12	243.0 (129.0-308.0)	18.15	
TT	0	-	-	
Control				
rs6313				
TC	7	220.7 (185.0-262.0)	10.44	0.54
CC	7	243.1 (180.0-334.0)	20.87	
TT	6	257.3 (166.0-374.0)	35.02	
rs6314				
TC	5	280.2 (189.0-374.0)	36.10	0.07
CC	15	226.9 (166.0-334.0)	11.39	
TT	0	-	-	

P-value based on ANOVA and Student's t-test.

serotonin levels were the first biomarker identified in autism research. It has been demonstrated that the serotonin concentration in the blood of autistic children is by 25-50% higher than in healthy subjects [37]. Many studies have contrasted blood 5-HT levels in autistic patients and controls depending on measurement method. Significantly higher 5-HT levels in about 30% autistic patients compared to controls were recorded both in whole blood, and in platelet-rich plasma, but not in platelet-poor plasma [38]. Moreover, Kolevzon et al. [6] did not found significant associations between serotonin level and the primary behavioral outcome measures in autistic patients.

In addition to the fact that we did not find any statistical difference in the groups, we also did not notice the relationship between the level of serotonin and rs6313 and rs6314 polymorphisms (Table 5). Some research suggests that serotonin levels depend on age. Our study group (ASD and control), with serotonin concentration level tests was homogenous in the age range of, 6-9

years. According to Chugani et al. [39] the brain serotonin synthesis increases up to the age of 15 and is 1.5 times higher in children with autism, than in adults. In healthy children, up to the age of 5, the serotonin level is twice as high, and then decreases and is close to the level observed in adults. Besides the fact that the mechanistic significance of the lack of association between serotonin and serotonergic neurotransmission and ASD development is obscure, also the widely accepted contribution of the serotonin system to ASD pathophysiology remains to be incompletely understood. The serotonin system is a candidate for involvement in ASD due to its multiple roles in brain system [29]. Whitaker-Azmitia and Azmitia [40] suggested that as a result of high levels of serotonin in children under 2 (when formation of blood-barrier is not yet complete) enter the developing brain and cause inhibition of 5-HT bearing neurons by a negative feedback mechanism of 5-HT.

Serotonin is a known metabolite of the dietary amino acid tryptophan and this notion supports a suggested role of diet as a potential therapeutic strategy in ASD, although underlying mechanisms are still poorly defined [41]. The suggested mechanistic links between the serotonin system, food-derived compounds and oxidative stress in ASD provide a rationale to further study the possible use of dietary interventions in ASD [42].

In conclusion, this study besides having certain limitations with a small sample size, show that **HTR2A** is clearly involved in the development of ASD in the Caucasian population. In addition, this study proved that genetic analysis focusing on defined gene SNPs in the *HTR2A* gene, rather than concentration of serotonin, can be used to determine the relevance of the serotonin receptor gene that affects the development and enhancement of ASD symptoms.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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