Original Article

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Angiotensin-converting enzyme genetic variants do not influence response to risperidone in autistic children

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Abstract

Genetics has been found to have a prominent role in autism and therefore pharmacogenetics may guide us to a better management of this disorder. Given the importance of Renin-Angiotensin System (RAS) in the function of the brain and its possible association with autism, genetic variations of RAS may influence response to autism treatment. In this study, 83 autistic children were enrolled (3 to 12 years of age). Degree of autism was confirmed by the DSM-V criteria and response to treatment was measured according to Aberrant Behavior Checklist (ABC) scale at baseline, 4 and 12 weeks of risperidone therapy. Polymorphisms (ACE I/D, rs4343 and rs4291) were determined by PCR-RFLP. Our results indicate the positive role of long term therapy in autism (12 weeks vs 4 weeks). The highest response rate in ACE ID gene was in the DD genetic variant at both 4 and 12 weeks of treatment. For the ACE A2350G gene, all genetic variants did not respond well to treatment at 4 weeks, however at 12 weeks, positive response was dominant in the AG genetic variant. Highest response rate in the ACE A240T gene belonged to the AT variant at both 4 and 12 weeks of treatment. However, our results indicate no significant association between ACE gene polymorphisms and response to risperidone therapy in autistic children based on ABC scaling. In conclusion, this study does not support the hypothesis of involvement of RAS genetics in response to risperidone in autistic children.

Keywords: Autism, Renin-angiotensin system, Angiotensin-converting enzyme, Genetic polymorphism, Single nucleotide polymorphism.

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1. Introduction

Autism is a complex neurodevelopmental disorder characterized by impaired social interaction, mutual communication skills and restricted and repetitive behavior (1). The prevalence of the disease is rapidly increasing and is reported to be 1 in every 54 children in the United States (2)

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and 0.1% in Iran according to a recent report (3). The main cause of autism has not yet been fully determined but it has long been presumed that it results from an interaction of both genetic and environmental factors (4). The higher concordance rate in monozygotic twins compared to dizygotic ones, clarifies the significant impact of genetics in this disorder (5-9). Although the importance of genetics in autism has been emphasized in many studies, still 70% of autism cases remain with an unknown genetic etiology (10).

Behavioral therapy has been introduced as the main approach to treatment of autism but due to the unavailability of trained specialists and high cost of services, pharmacotherapy has always been a complimentary choice in approach to such patients (11). Many psychotropic agents have been studied for the treatment of autism related symptoms amongst which risperidone has shown promising results (11-13). Risperidone, an atypical antipsychotic, helps mitigate aggressive behavior and irritability, self-injuries, stereotypic behavior, social withdrawal and lack of interest in autistic patients (14, 15). As it is known, patients respond differently to same doses of the same drug. When treating autistic patients with atypical antipsychotics, only 30-70% achieve favorable therapeutic response (16). Along with numerous factors contributing to variations in drug response, genetics is estimated to be one of the most important factors accounting for 20-95% of the variability in drug effects (17). There is not much evidence regarding genetic factors that underlie this inter individual variation in response to treatment in autism.(18). In this regard, the special and pivotal role of applying pharmacogenetics in psychiatric disorders is highlighted which will lead to smarter selection of the patients and their treatment (19, 20). Risperidone binds with strong affinity to several neurotransmitter receptors in the brain mainly antagonizing the effect of dopamine and serotonin (21).

Angiotensin II (Ang II), the ultimate product of renin angiotensin system (RAS), serves as a non-classical neurotransmitter that also interacts with the mentioned neurotransmitters and is found responsible for important functions in the brain (22-24). It has also been proposed to have potential role in the incidence of neurological and psychiatric diseases by controlling emotions, cognition and movement through modulating dopaminergic neurotransmission (25-27). Ang II is formed from angiotensin I via the catalytic action of angiotensin converting enzyme (ACE) and applies its physiological effects principally through AT1 and AT2 receptors (28). Earlier studies suggest that certain polymorphisms on ACE gene such as ACE I/D, rs 4343 and rs 4291 may affect serum ACE activity and as a result the level of Ang II (26, 29). On the other hand, significant association between genetic variants of ACE and autism has been previously reported (30).

According to the above explanations and the fact that genetic differences are influential in both the autism incidence and its treatment results, we aim to investigate the possible effect of genetic differences in ACE I/D, rs 4343 and rs 4291 and their related haplotypes in response to risperidone therapy in autistic children.

2. Materials and methods

2.1. Study population

Eighty-three autistic outpatient participants (age: 3 to 12) referred to Imam Reza Clinic affiliated to Shiraz University of Medical Sciences were enrolled in this cross-sectional study taking place from 2013 to 2015. Diagnosis of autism was based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) criteria (31) and confirmed by a child psychiatrist. Condition of patients was regularly followed for at least 1 year by the same psychiatrist. Children with other mental or psychiatric disorders that may have needed treatment according to their diagnosis, were excluded from the study. The severity of autism at baseline and the effectiveness of treatment at specified time points were assessed using Aberrant Behavior Checklist (ABC)(32) rating scale which consists of 58 questions divided into 5 subscales including irritability, lethargy and social withdrawal, stereotypic behavior, hyperactivity/noncompliance, and inappropriate speech (33). The ABC scoring sheet was completed by the pharmacist with the guidance of the child's parent or caregiver and under the supervision of the psychiatrist. Scoring for each question was in the

range of 0-3, 0 indicating "no problem" and 3 being "severely problematic". Response to treatment was considered as the outcome and was evaluated based on changes in ABC score compared to baseline evaluations.

The work was approved by the local committee of ethics of medical experiments on human subjects of Shiraz University of Medical Sciences with the ethical code of 1396-01-05-15228 and was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and Uniform Requirements for manuscripts submitted to biomedical journals. Informed written consent (approved by the Institutional Ethical Committee) was attained from parents/legal representatives of all children included in the study.

Risperidone was given at home by the children's parents. The dose of risperidone was titrated up to 2 mg/day starting with 0.5 mg as the starting dose and increasing the dose with 0.5 mg increments weekly for the first 3 weeks for children weighing 10 to 40 kg and 3 mg/day as a final dose for children weighting above 40 kg with the same dose titration manual (34).

It should be noted that these participants had not taken any other neuroleptics or any psychotropic medicines within the past six months of taking part in this study. Before beginning treatment and 4 and 12 weeks after initiation of treatment, severity of autism was assessed using ABC rating scale. Patients were divided into two groups of treatment-responsive and non-responsive based on ABC score. The extent of response to treatment based on ABC scores at various treatment durations (1 month and 3 months) was evaluated based on a 50% reduction in at least 2 subscales of the ABC checklist with no other subscale indicating a 10% or more increase in the severity of symptoms.

2.2. DNA Extraction & Genotype Determination

Genomic DNAs were extracted from blood leukocytes, using the salting out method (35). The extracted DNAs were dissolved in sterile distilled water and stored at 4 °C for further PCR analysis. PCR amplification/detection of polymorphisms was carried out using a previously introduced protocol (36, 37). Primers are listed in Table 1. Briefly, 100-200 ng of genomic DNA was amplified in 15 µl of 1 × PCR master mix (67 mM Tris base, pH 8.8, 16.6 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$, 0.1 % Tween-20, 200 µM dNTPs, 5 % glycerol, 100 µg/ml cresol red) containing 0.2-2.0 µM of each primer and 0.5 U of Taq DNA polymerase (Cinnagen Inc., Tehran, Iran). After initial denaturation at 96 °C for 2 min, PCR was performed for 5 cycles, each one comprised of denaturation at 96 °C for 40s, annealing at 60 °C for 50s and extension at 72 °C for 30s followed by 25 cycles of denaturation at 96 °C for 40s, annealing at 55 °C for 50s and the extension at 72 °C for 30s. PCR products (7 µl) were digested with the specified enzymes mentioned in Table 1. Briefly, as for A-240T (rs4291), PCR products were digested using XbaI NEB enzyme and incubated at 370C overnight. Digestion of PCR products for A2350G (rs4343) was performed using BstU1 NEB enzyme and incubated for 24 hrs at 650C. Digested fragments were separated by electrophoresis on 3% agarose (Invitrogen® UltraPure) gel after an overnight incubation (Table 1). After being stained with ethidium bromide, fragments were visualized

Table 1. Primers for ACEI/D, A2350G and A-240T polymorphisms and DNA fragment sizes (37).

Variant	Primer sequence	Allele	DNA fragment size
ACE I/D	F-CTG GAG ACC ACT CCC ATC CTT TCT	I	490
	R-GAT GTG GCC ATC ACA TTC GTC AGA T	D	290
	F-TTT GAG ACG GAG TCT CGC TC	I	408
	R-GAT GTG GCC ATC ACA TTC GTC AGA T		
A-240T	F-TCG GGC TGG GAA GAT CGA GC	A	137
	R-GAG AAA GGG CCT CCT CTC TCT	T	114+23
A2350G	F-CTG ACG AAT GTG ATG GCC GC	A	122
	R-TTG ATG AGT TCC ACG TAT TTC G	G	100+22

Table 2. Demographic data of autistic patients (N=83).

Age mean ± SD (years)		7.5±2.8
Male/Female (%)		59/24 (71.1/28.9)
BMI mean ± SD (kg/m2)		17.1±4
Educational Status	Not going to school	32 (38.5)
	Going to autistic school	41 (49.4)
	Going to ordinary school	10 (12.0)
Comorbidity (%)	ADHD	21 (25.3)
	Seizure	5 (6.0)
	CP	3 (3.6)
	Down syndrome	2 (2.4)
	Deafness	4 (4.8)
	Tic	1 (1.2)
	Brain tumor	1 (1.2)
	Diabetes	1 (1.2)
	None	46 (55.4)

BMI: Body Mass Index; ADHD: Attention Deficit Hyperactivity Disorder; CP: Cerebral Palsy.

in a UV transilluminator. For confirmation, all of the samples were genotyped at least twice.

2.3. Statistics

SPSS Statistics 16.0 software was used for statistical analysis. Hardy-Weinberg equilibrium (HWE) was calculated for the distributions of genotypes using Chi-square test and by means of Arlequin 3.1 software package. Continuous variables are presented as mean±SD. Genotype frequencies are shown in percentage (%). Distribution of all continuous variables was tested for normal distribution. Chi-square test was used to compare ABC scores after 4 and 12 weeks of treatment in order to determine association between response to risperidone with respect to genotypes and haplotypes. General linear model was used for evaluating association of treatment duration with response to therapy. P value< 0.05 was considered statistically significant.

3. Results

Demographic characteristics of enrolled patients are demonstrated in Table 2. As shown in Table 3, effectiveness of treatment with risperidone is observed comparing baseline ABC values with ABC at 4 and 12 weeks of treatment (P values<0.001). Also the positive impact of long term treatment (12-week vs 4-week) is evident according to results demonstrated in Table 3. According to our results based on ABC scoring system, 27.7% and 50.6% of patients were responsive to treatment respectively at 4 and 12 weeks of therapy. Table 4 demonstrates ACE genetic haplotype frequencies in our study population and their association with achieving response to risperidone according to ABC questionnaire at 4 and 12 weeks of therapy. No significant association was observed in ABC score reduction (responsiveness) with respect to different haplotypes. Also ACE genetic

Table 3. Efficacy of autism treatment with risperidone at 4 weeks and 12 weeks according to ABC score. (N=83).

Autism severity	mean ± SD	Comparison	P-value
ABC0	49.12 ± 23.363	ABC0 vs ABC1	<0.0001
ABC1	39.40 ± 24.086	ABC0 vs ABC3	<0.0001
ABC3	36.49 ± 24.560	ABC1 vs ABC3	<0.0001

ABC0: ABC-C score at baseline; ABC1: ABC score at week 4; ABC3 ABC score at week 12.

Table 4. The probable association between ACE genetic haplotypes and response to risperidone in 83 autistic patients according to ABC scoring system.

Haplotypes in risperidone users n=83		ABC1		P value	ABC3		P value
		responsive n(%)	non-responsive n(%)		responsive n(%)	non-responsive n(%)	
DTG	Positive n=1	0	1 (1.2)	1.000	0	1 (1.2)	0.494
	Negative (n=82)	23 (27.7)	59 (71.1)		42 (50.6)	40 (48.2)	
IAA	Positive (n=16)	2 (2.4)	14 (16.9)	0.213	7 (8.4)	9 (10.8)	0.542
	Negative (n=67)	21 (25.3)	46 (55.4)		35 (42.1)	32 (38.5)	
DAG	Positive (n=13)	6 (7.2)	7 (8.4)	0.173	8 (9.6)	5 (6.02)	0.39
	Negative (n=70)	17 (20.5)	53 (63.8)		34 (40.9)	36 (43.4)	
DAA	Positive (n=47)	13 (15.6)	34 (41.0)	0.99	25 (30.1)	22 (26.5)	0.59
	Negative (n=36)	10 (12.0)	26 (31.3)		17 (20.5)	19 (22.9)	
IAG	Positive (n=3)	1 (1.2)	2 (2.4)	1.000	0	3 (3.6)	0.116
	Negative (n=80)	22 (26.5)	58 (69.9)		42 (50.6)	38 (45.8)	
DTA	Positive (n=3)	1 (1.2)	2 (2.4)	1.000	2 (2.4)	1 (1.2)	0.509
	Negative (n=80)	22 (26.5)	58 (69.9)		40 (48.2)	40 (48.2)	
ITA	Positive (n=1)	0	0	-	0	0	-
	Negative (n=82)	23 (27.7)	60 (72.3)		42 (50.6)	41 (49.4)	
ITG	Positive (n=1)	0	0	-	0	0	-
	Negative (n=82)	23 (27.7)	60 (72.3)		42 (50.6)	41 (49.4)	

ABC1: ABC score at week 4; ABC3 ABC score at week 12.

variants, their frequencies in our patients and the relation between the variants and responsiveness to risperidone therapy were evaluated based on ABC scoring system (Table 5). Although no significant association was observed, the highest response rate in ACE ID gene was in the DD genetic variant accounting for 87% and 83% of responsive cases of the genetic variant at 4 and 12 weeks of treatment respectively. For the ACE A2350G gene, all genetic variants did not respond well to treat-

Table 5. The relationship between ACE genetic variants and response to risperidone in 83 autistic patients.

Genes in risperidone users n=83		ABC1		P value	ABC3		P value
		responsive n(%)	non-responsive n(%)		responsive n(%)	non-responsive n(%)	
ACE ID	II	2 (2.4)	4 (4.8)	0.18	3 (3.6)	3 (3.6)	0.354
	ID	1 (1.2)	12 (14.4)		4 (4.8)	9 (10.8)	
	DD	20 (24.1)	44 (53)		35 (42.2)	29 (34.9)	
ACE A2350G	AA	8 (9.6)	17 (20.5)	0.208	11 (13.2)	14 (16.8)	0.601
	AG	8 (9.6)	33 (39.7)		23 (27.7)	18 (21.7)	
	GG	7 (8.4)	10 (12.0)		8 (9.6)	9 (10.8)	
ACE A240T	AA	7 (8.4)	17 (20.5)	1.00	11 (13.2)	13 (15.6)	0.858
	AT	15 (18.1)	40 (48.2)		29 (34.9)	26 (31.3)	
	TT	1 (1.2)	3 (3.6)		2 (2.4)	2 (2.4)	

ABC1: ABC score at week 4; ABC3 ABC score at week 12.

ment at 4 weeks, however at 12 weeks of treatment responsive patients were dominant in the AG genetic variant. Highest response rate in the ACE A240T gene belonged to the AT variant at both 4 and 12 weeks of treatment.

It is noteworthy that all genotypes were in HWE (P>0.05).

4. Discussion

Roughly 800 genes are proposed to be involved in synaptic function and cortical development which are linked to autism (38). Recent developments in psychiatric research highlight the role of neuro-inflammation in many disorders such as autism (39, 40). The significant neuro-inflammatory role of Ang II as the main product of RAS and its impact on cognitive disorders (27, 41) lead us to the hypothesis that genetic variations of RAS might contribute to different response rates to treatment in autistic patients. This idea is strengthened by the results of a report revealing a significant association between polymorphisms of ACE and autism (30). Although no study to date has investigated this specific polymorphism and its association with response to autism treatment, DRD3 genetic variants have been associated to good response rates in Iranian autistic children treated with risperidone (42).

A recent Review focused on the play of genetics in autism in the Middle Eastern countries emphasizing on their influence on early detection of the illness and proposing various genes as potential therapeutic targets in the treatment of autism. Although the report focused on data of Middle Eastern countries, genes involved seemed heterogenous and not uniform in different areas. Therefore, like many other illnesses, ethnicity plays a pivotal role in detection of predisposing genes as well as response to treatment in autism (43). As reported previously, GG variant of rs4343 is associated with increased production of Ang II (30). Several explanations can enlighten the involvement of RAS in treatment response of autism. Brain-derived neurotrophic factor (BDNF) with a major role in synaptic connections' plasticity, neuronal survival and regulating differentiation of phenotype in mature neurons, is widely expressed in the mammalian brain (44). Higher levels of BDNF has been detected in both the periphery and brain tissue of the autistic children in comparison to general population (45, 46). Alongside, Ang II, the main product of RAS, vastly upregulates BDNF gene expressions (47). BDNF is suggested to be involved in regulating the hypothalamic-pituitary-adrenal (HPA) axis. Stress and depression alter BDNF expression in areas of the brain related to the activation of HPA axis (48), which is disrupted in autism (49, 50). Risperidone, as one of the main treatments in autistic patients, is suggested to significantly decrease synthesis and release of BDNF in some brain areas (51). Association of a genetic variant on BDNF gene with clinical response to risperidone is also reported (52). However in this report this genetic variant failed to show associations with improved response rate in risperidone treated autistic children.

Inflammatory biomarkers have been found to be significantly higher in Autistic patients, which influence the permeability of blood brain barrier and induce neuro-inflammation (40, 53). Substantial cross-talk between RAS and the immune system exists. Ang II significantly induces production of IL-6, an inflammatory marker, in cardiac fibroblasts (54) as well as brain astrocytes (55). Blockade of Ang II receptors, reverses inflammation in the brain (41, 56). Moreover prenatal exposure to IL-6 changes RAS activity emphasizing on the interaction between immune system and RAS. Studies have reported that antipsychotics such as risperidone, modulate inflammatory cytokines, which can be related to symptom improvement in psychosis (57) and also an explanation to the mechanism involved in the improved ABC score following risperidone treatment in our study population but at the same time without any specific genetic variant orientation.

Another mechanism by which Ang II can be associated to the pathophysiology of autism, is oxidative stress (58). Since the brain has very little capacity in scavenging free radicals, high levels of Ang II, as a pro oxidant agent, makes a person more vulnerable to upcoming damages (59). It is reported that risperidone significantly lowers oxidative stress (60, 61). It can be assumed that risperidone might reduce oxidative stress by means of suppressing Ang II.

Regarding neurotransmitters, autism is among the hyperdopaminergic disorders. Dopamine synthesis is influenced by Ang II in the brain (24) and risperidone induces its pharmacological action by antagonizing dopamine receptors; therefore it can be presumed that the dopamine lowering effect of risperidone may be induced via decrease in Ang II levels and thus the genetic variants of the RAS pathway might prove to have a prominent role in response to therapy.

All the above mechanisms explained may be considered as responsible mechanisms for the impact of the RAS system and Ang II on the efficacy of risperidone treatment in autistic children, however in the Iranian population of this study, variation of genetics of the ACE gene failed to be a prominent factor affecting response. But since no other similar study to date has taken place in other autistic populations, comparison of our results was quite impossible and further studies in other ethnic populations is mandatory due to genetic variations in different study populations.

5. Conclusion

The proposed neuro inflammatory role and oxidative actions of Ang II along with the influence of neurotransmitters in autism, their interactions with RAS and the effect of risperidone on the mentioned systems, are all suggested proof to justifying our hypothesis regarding involvement of RAS in the treatment of autism. Yet, this is the first study reporting the evaluation of such probable association and must be replicated in other populations. Considering major contribution of RAS in regulating local immune response and tissue homeostasis, we believe further research is required to guarantee the role of RAS in the treatment of autism.

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Conflict of Interest

None declared.

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