Transforming growth factor beta 1 869T/C and 915G/C polymorphisms and risk of autism spectrum disorders

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Abstract

Background: Transforming growth factor-β1 (TGF-β1) has been found to play a crucial role in early central nervous system development. Several studies have illustrated decreased TGF-β1 levels in sera and brains of autistic children. Two point mutations in the TGF-β1 signal peptide at 869T/C and 915G/C have been reported to influence TGF-β1 expression. The aim of the present study was to investigate the correlation of TGF-β1 polymorphisms and their haplotypes with autism.

Methods: This study was performed on 39 autistic patients and 35 age- and sex-matched normal controls in an Iranian population, using the sequence specific primed-polymerase chain reaction (PCR-SSP) technique. Patients were divided into mild-to-moderate and severe groups according to the childhood autism rating scale.

Results: No significant differences were observed for allele, genotype, or haplotype frequencies between the autistics and controls. Only a slight difference was observed in GC25 between the controls and all children with autism.

Conclusion: Thus, these results indicate that the polymorphisms in TGF-β1 gene may not play an important role in the development of autism.

Keywords: Autism spectrum disorders, Development, Polymorphism, Transforming Growth Factor beta 1,

Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by difficulties in social interactions and communication, and repetitive and stereotyped patterns of behaviors and interests with various levels of severity occurring before three years of age (1). Although the exact cause of these disorders remains poorly understood, immunological factors have been proposed to have a major role in their pathophysiology (2). Several studies have shown a correlation between immune system abnormalities and autism spectrum disorders. These abnormalities include inappropriate immune regulation resulting in abnormalities in the functional immune cell subsets and autoimmunity such as autoantibodies generated against the central nervous system (CNS) (2-4). It is assumed that aberrant immune responses during the critical neurodevelopmental period may result in the development of neurological disorders (5). The plausibility of this hypothesis with regard to immune

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system abnormalities in autistics has been derived from the pivotal role of the immune system in neurodevelopment and the ability of these alterations to influence the CNS (5). Both the immune and nervous systems are highly evolved systems that cross talk via cytokines and neuro-mediators such as neuropeptides (6, 7).

Transforming growth factor-β1 (TGF-β1) is an important immune regulator critical for immune homeostasis. Accumulating evidence suggests that TGF-β1 has a crucial regulatory role in CNS development and potential implications for neurogenesis in a variety of TGF-β1-related CNS diseases (5, 8). TGF-β1 knockout mice were shown to have severe cortical developmental impairment with pervasive increased neuronal cell death and microgliosis complication (9). Furthermore, decreased levels of TGF-β1 in serum have been observed in autistic children (10, 11). These reports suggest that immune system aberrations may lead to abnormal immune responses, autoimmunity, or adverse neuroimmune interactions during brain development.

The human TGF-β1 gene, located on chromosome 19q13.1–3, contains five described polymorphisms: two in the promoter region at positions 800 G/A and 509 C/T and three located in the coding sequence at positions 869 T/C, 915 G/C and 1628 C/A (12-15). The point mutations at positions 869 T/C and 915 G/C result in amino acid substitutions in the signal peptide at codons 10 (Leu/Pro) and 25 (Arg/Pro), respectively. It has been found that these polymorphisms genetically control TGF-β1 serum concentrations. Moreover, the presence of leucine and arginine at codons 10 and 25 determines the high-producer genotype, while proline at both codons is associated with relatively lower levels of TGF-β1. However, there are some conflicting results concerning codon 10 polymorphisms. Some studies demonstrated that leucine is a high producer (14), while others (15, 16) reported that proline at codon 10 results in the high-producer genotype. However, there is no controversy that the polymorphisms at codons 10 and 25 affect TGF-β1 production. In the present study, two coding polymorphisms within exon 1 of the TGF-β1 gene at positions 869 T/C and 915 G/C were analyzed to determine the prevalence of particular TGF-β1 genotypes in autistic children. Aberrations in immune

system regulation or impairment in immune homeostasis may result in chronic inflammation, autoimmunity, or inappropriate immune responses. These may cause inflammation in the CNS or brain leading to altered neurodevelopment. In addition, TGF-β1 is a potent immunosuppressive cytokine as well as a crucial regulator in brain development (5). Furthermore, it is believed that TGF-β1 protects the brain from neuronal degeneration during inflammation in the CNS (17-19). Therefore, TGF-β1 has been widely recognized as a cytokine that responds to brain injury. Several studies have demonstrated altered TGF-β1 levels in brains and sera of autistics (3, 11). It has also been shown that the 509C/T and 869T/C point mutations in TGF-β1 lead to altered TGF-β1 production and/or activity, which may modulate an individual's susceptibility to autism. Given the key role of these polymorphisms in cytokine production and the low TGF-β1 serum levels in autistics, we investigated whether TGF-β1 polymorphisms are risk factors for the development of autism. Our research focused on point mutations involved in TGF-β1 production; namely alleles T (leucine) and C (proline) at codon 10 and alleles G (arginine) and C (proline) at codon 25.

Materials and Methods
Patients
Thirty-nine autistic children, aged 7-13, were included in this study. The Childhood Autism Rating Scale (CARS) was used to confirm the diagnosis of autism and assess its severity. A 29.5 cut-off point was employed to diagnose autism. Scores of 30–36.5 and 37–60 were classified as mild-to-moderate and severe, respectively. In addition, criteria from the Diagnostic and Statistical and Manual of Mental Disorders 4th Edition (DSM-IV) were also used to differentiate autistic children with CARS > 30 from other developmental disorders e.g. Rett, Asperger's, and childhood disintegrative disorder (20). The control group included 35 age-matched, healthy children with IQs of 90-110. None of the children in the control group had neurological or psychological deficits. Informed consents were obtained from parents in both groups. The study design was in accordance with the tenets of the Helsinki Declaration, and was approved by the Research Ethics Committee of Azad University of Medical Sciences, Mashhad Branch. Demographic

data, previous medical histories, and clinical signs and symptoms of all children were also obtained.

PCR Method

Peripheral blood was obtained and genomic DNA extracted by the salting-out method as described previously (21). To determine TGF-β1 genotyping at codons 10 (T/C) and 25 (G/C), sequence-specific primed-polymerase chain reaction (PCR-SSP) was performed. The following primers were used to amplify codons 10 (T/C) and 25 (G/C):

5′-CGGGCTGCGGCTGCTGCC-3′ (T10),

5′- CGGGCTGCGGCTGCTGCT -3′ (C10),

5′-TTTCGTTGTGGGTTTCCACCATTAG-3′
(common codon 10) and

5′-GTGCTGACGCCTGGCCG-3′ (G25),

5′- GTGCTGACGCCTGGCCC -3′ (C25),

5′-GGCTCCGGTTCTGCACTC-3′ (common codon 25). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was amplified as an internal control for the genomic DNA preparation. The optimized PCRs were performed in 20 μl reactions containing 50 ng of genomic DNA, 10 nmol of specific and control primer mixes, 200 μM of each deoxynucleotide triphosphate (dNTP), 1x ammonium-sulfate-based PCR buffer, 1.5 mM MgCl2, 5% DMSO, and 0.5 U of HS-Taq DNA polymerase (Parstous, Iran). Amplifications were performed on a Corbett Research Thermocycler (Corbett, Australia), using the following conditions: initial denaturation at 94 ℃ for 10 min followed by 35 cycles of 30 s at 94 ℃, 20 s at 65℃ for 869T/C and 30 s at 63 ℃ for 915G/C, 30 s at 72 ℃ and a final extension at 72 ℃ for 5 min. The PCR products were analyzed in 2% agarose gel stained with green viewer and visualized under UV.

Statistical analysis

All statistical analyses were performed using SPSS version 11.5 (SPSS Inc. Chicago, IL, USA). Genotype and allele frequencies were compared between the study groups by χ^2 test, Fisher's exact test and odds ratios (OR) with 95% confidence intervals (CIs). Demographic and clinical data between groups were compared by χ^2 test and Student's t-test. A p-value less than 0.05 was considered statistically significant.

Results

The demographic and clinical characteristics of all groups are summarized in Table 1. The genotype and allele frequencies for the TGF-β1 polymorphisms in the autistics, which were divided into mild-to-moderate and severe, and controls are shown in Table 2. The genotype distribution among the autistics and controls was in Hardy–Weinberg equilibrium. In this study, two polymorphic positions within the TGF-β1 codon 10 T/C and 25 G/C were analyzed. No significant differences were observed in the distribution of TGF-β1 genotypes and allele frequencies between patients and controls. Only a slight difference was observed in GC25 between the controls (n = 35) and all children with autism (n = 39, p = 0.082). In addition, to investigate whether any specific haplotypes would relate to the development of autism, all possible haplotypes composed of these polymorphisms were examined (Table 3). The result showed that CC/CC (low producer) haplotype was not detected in any group. Moreover, the TC/CC low producer was detected in only one subject in the control group. The major haplotypes in both groups were those polymorphisms considered as high producers (TT/GG and TC/GG). Furthermore, no significant differences in TGF-β haplotypes were identified between autistic patients and controls (Table 3).

Discussion

In this study we focused on point mutations of alleles that were reported to be involved in TGF-β1 expression; namely alleles T (leucine) and C (proline) at codon 10 and alleles G (arginine) and C (proline) at codon 25. No significant differences were found in the distributions of TGF-β1 alleles, genotypes, or haplotype frequencies between autistics and controls.

Over the past decade, numerous reports have noted abnormalities or alterations of immune system activity in autistics; these include increased serum levels of inflammatory cytokines and factors such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and high sensitivity C-reactive protein (hsCRP) (22-25). It has been reported that autoantibodies against brain and CNS proteins exists in 30–70% of autistic patients (4, 26-28). In addition, decreases in lymphocytes and T cell mitogen responses, and an imbalance of serum immunoglobulin levels have been reported in a significant number of autistic children (2, 29). Overall, the data suggests that immune dysfunction and excessive inflammation play important pathophysiologic roles in autism disorders.

Table 1. Comparison of demographic and clinical data as well as abnormalities in CT-scans between autism (n = 39) and control (n = 35) groups (*Not significant).

Variables		Autism group	Control group	*p*-value
Mean age (Years) ± SD		8.54 ± 1.68	7.9 ±3.1	NS
Gender (boys/girls)		25/14	20/15	NS
BMI (kg/m2) ± SD		22.85 ± 2.19	21.14 ± 2.45	NS
Age of diagnosis		2.8	–	–
Severity (%)	Severe	29 (74.4%)	–	–
	Mild/moderate	10 (25.6%)	–	–
Obstetric complications (%)		4 (25%)	6 (37.5%)	NS
Another disease (%)		8 (20.5%)	2 (5.7%)	NS
Family history (%)		8 (20.5%)	0 (0.0%)	0.008
Epilepsy (%)		12 (30.8%)	0 (0.0%)	0.001
Hypoxia (%)		3 (7.7%)	0 (0.0%)	NS
Abnormal CT-scan (%)		1 (2.6%)	0 (0.0%)	NS

Table 2. Genotype and allele frequencies for the TGF-β1 Codon 10 and 25 polymorphisms in autistic patients and controls (*Fisher's exact test).

Genotype	Autism cases(n=39)		Controls (n=35) (%)	(*p*)*	OR *p*	OR (CI 95%)
	Mild/ Moderate 10 (%)	Severe 29 (%)				
Codon 10 T>C						
TT (%)	6 (60%)	14 (48.3%)	11 (31.4%)			1.0 (reference)
TC (%)	3 (30%)	10 (34.5%)	17 (48.6%)	(0.51)	0.162	0.324 (0.06- 1.57)
CC (%)	1 (10%)	5 (17.2%)	7 (20%)		0.258	0.262 (0.02- 2.66)
χ^2 **HW** (*p*)	0.4 (0.52)	1.62 (0.2)	0.008 (0.92)			
Allele						
T (%)	15 (75 %)	38 (65.5%)	39 (55.7%)	(0.26)		1.0 (reference)
C (%)	5 (25 %)	20 (34.5%)	31 (44.3%)		0.086	0.435 (0.16- 1.12)
Codon 25 G>C						
GG (%)	7 (70%)	20 (69%)	30 (85.7%)			1.0 (reference)
GC (%)	2 (20%)	9 (31%)	4 (11.4%)	(0.12)	0.082	3.05 (0.86- 10.73)
CC (%)	1 (10%)	0 (0%)	1 (2.9%)		0.942	1.11 (0.06-18.64)
χ2 **HW** (p)	1.40(0.23)	0.97 (0.32)	2.56 (0.10)			
Allele						
G (%)	16 (80%)	49 (84.48%)	64 (91.42%)	(0.27)		1.0 (reference)
C (%)	4 (20%)	9 (15.51%)	6 (8.57%)		.099	2.667 (0.83- 8.55)

Table 3. Distribution of TGF-β1 (codon 10 T/C and codon 25 G/C) haplotypes among autistic patients and healthy controls.

Production level	Haplotype	Control	Mild/ Moderate	Severe	χ^2 (p)
High	TT/GG	11 (31.4%)	5 (50%)	8 (27.6%)	
	TC/GG	14 (40%)	2 (20%)	8 (27.6%)	
Intermediate	TT/GC	0 (0.0%)	0 (0.0%)	6 (20.7%)	
	TC/GC	2 (5.7%)	1 (10%)	2 (6.9%)	7.14 (0.128)
	CC/GG	5 (14.3%)	0 (0.0%)	4 (13.8%)	
Low	TT/CC	0 (0.0%)	1 (10%)	0 (0.0%)	
	TC/CC	1 (2.9%)	0 (0.0%)	0 (0.0%)	
	CC/GC	2 (5.70%)	1 (10%)	1 (3.4%)	
	CC/CC	0 (0.0%)	0 (0.0%)	0 (0.0%)	

TGF-β1 is considered to be one of the critical immunosuppressive cytokines in immune homeostasis and T cell activated unresponsiveness (30-32). Furthermore, evidence suggests that during the brain development, glial and neuronal cells produce TGF-β1, which plays a crucial role in the regulation of early CNS development such as astrocyte differentiation (33, 34), synaptogenesis (35), neuronal migration in the cerebral cortex (36), neuronal survival (37, 38), neuronal death, microgliosis control (9), wound healing, and immunosuppression (39).

Recently, in accordance with several other publications, we found (unpublished data) that serum levels of TGF-β1 are significantly lower in autistics than in age and gender-matched controls (10, 11). These findings are consistent with the hypothesis that reduced levels of this cytokine may lead to an inappropriate regulation of immune responses as well as the development of neuroinflammation disorders such as autism spectrum disorders. However, it is not yet demonstrated that reduction of TGF-β1 is a primary cause of autism or simply a secondary reflection of the disorder.

Given the key role of TGF-β1 in brain development and inflammation, we investigated the association between TGF-β1 gene polymorphisms and autism. Consequently, estimated alleles, genotypes, and haplotypes frequencies were compared between autistic patients and normal controls in an Iranian population. We found no association between the TGF-β1 gene polymorphisms and autism. It is well established that polymorphisms at 509C/T and 869T/C influence TGF-β1 production and resultant serum levels. It has also been demonstrated that the TT/GG and TC/GG haplotypes are associated with high TGF-β1 expression. The TC/GC, CC/GG, and TT/GC haplotypes are associated with intermediate expression, and the CC/GC, CC/CC, TT/CC and TC/CC haplotypes are known as low expressors. Based on the relatively low TGF-β1 expression in our study (unpublished data), we predicted that the subjects in our study with the CC/GC, CC/CC, TT/CC, TC/CC polymorphisms would be low expressors. Surprisingly the CC/CC and TC/CC polymorphisms were not detected in autistics, and only the TC/CC polymorphism was detected in one control subject. Our results also demonstrated that the dominant haplotypes in autistics were TT/GG and TC/GG, which contribute to high and intermediate expressors, respectively. Therefore, it seems difficult to assess only the influence of different genotype variants for the TGF-β1 serum levels in autistics. On the other hand, considering the relatively high rates of autoimmunity and inflammatory diseases in autistics and their families (40), decreased TGF-β1 levels in sera and brains may be explained by other factors such as defects in regulatory T cell development. Regulatory T cells are responsible for TGF-β1 production, self-tolerance, and immune homeostasis.

In conclusion, we found no association between autism and TGF-β1 gene polymorphisms in codons 10 and 25. Considering the influence of polymorphisms in TGF-β1 expression, further studies may be required to determine the effects of decreased levels of TGF-β1. More importantly, due to the small sample size, we believe the findings of the present study should be tested with a larger number of patients. Undoubtedly, more findings should be taken into account in the assessment of autism and these will help us to understand the underlying clinical and molecular mechanisms of the disorder.

Acknowledgements

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