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Comparison of Serum USP9x and TGF-β Levels in Children with Autism Spectrum Disorders with Healthy Controls

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ABSTRACT

Introduction: USP9X has been associated with neurodevelopmental disorders due to its role in synaptic development and neural function. This study aimed to compare USP9X and TGF-β levels in children with autism and healthy controls, and explore their relationship with autism severity.

Methods: Serum USP9X and TGF-β levels were measured in 41 healthy control children (aged 3–12 years) and 41 children with autism.

Results: Our study revealed a significant increase in USP9X levels (p=0.001) among children with autism compared to controls. However, TGF-β levels showed no significant difference between the two groups. Furthermore, we observed a positive correlation between difficulty in making eye contact subscale and blood levels of both USP9X and TGF-β.

Conclusions: This study is the first to compare serum USP9X levels in children with autism to healthy controls. Our findings suggest USP9X's potential role in autism development, emphasizing the need for further research on its involvement in neurodevelopmental processes.

Keywords: Autism, USP9X, TGF-β, Etiology

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by a lack of social skills, verbal and nonverbal communication impairments, and limited and stereotypical interests and activities. Males are four times more likely than females to have it (1). Recent estimates from the Centers for Disease Control and Prevention (CDC) show that the prevalence of autism among American children has increased to one in 36, or around 2.8%, from one in 44 (2.3%) two years ago (2). Even though the etiology of autism is multifactorial, research points to a strong genetic component in the etiology of autism. Mutations or variations in certain genes involved in synaptic function and neuronal connectivity have been associated with an increased risk of developing autism (3).

Highlights

- **We investigated USP9X and TGF-β levels in children with autism and healthy children.**
- **Our study is the first study evaluating serum USP9X levels in autism.**
- **TGF-β levels did not show a significant difference between the two groups.**
- **USP9X levels were significantly higher in the group with autism.**
- **The relationship between USP9X and autism may be an important research topic.**

Recent studies suggest that protein cycling may play an important role in ASD (4). Protein homeostasis is a mechanism by which cells maintain physiological protein levels and abnormal neuronal homeostasis leads to abnormal protein levels and disorders associated with excessive protein aggregation. One of the main mechanisms of homeostasis is ubiquitylation, which is protein degradation by the ubiquitin-proteasome system; the other is the deubiquitylation system, which opposes this process by removing ubiquitin from proteins (5). Ubiquitylation is a post-translational modification caused by the addition of ubiquitin, which consists of 76 amino acids, to the protein structure. In cells, the ubiquitin system is responsible for the degradation of a large number of short-lived proteins. The covalent binding of proteins to ubiquitin ensures that they are at the target point for degradation. Many activities such as cell cycle, signal transduction, transcriptional regulation, receptor-mediated regulation, synaptic remodeling, and endocytosis are regulated

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by the ubiquitin system (6). Due to the close connection between the ubiquitin system and proteins, disruption of this system can cause neurodevelopmental disorders (7).

Deubiquitination is a process in which deubiquitinating enzymes remove ubiquitin from ubiquitinated proteins and reverse the ubiquitination event (8). Deubiquitile enzymes (DUB) invert the ubiquitin-substrate protein relationship by inhibiting the binding of ubiquitin to the substrate protein. The human genome encodes around 95 DUB. A substrate-specific protease (USP) is the largest class, and it governs cellular activities precisely (9). Usp9x is a large DUB (2554 amino acids)(10). USP9X is a highly conserved X-chromosome gene that encodes a deubiquitylating enzyme (DUB). The X-linked USP9X gene encodes a structurally and functionally conserved deubiquitinating enzyme with a UBL (ubiquitin-like) domain and a catalytic ubiquitin specific protease (USP) domain (11). Usp9x protein is localized in synapses in rats, indicating its function in mammalian synaptic development (12). It is known to have an important function in both human and rat neural development and is essential for fetal development. USP9X expression is highest during embryogenesis (11). Even when USP9X is reduced in the adult central nervous system, it continues to be strongly expressed in neurogenic regions such as the sub-ventricular zone of the lateral ventricles and the sub-granular zone cells of the dentate gyrus (13).

Several important substrates of USP9X control processes related to homeostasis and/or brain development. These include elements of the Notch, Wnt and Transforming Growth Factor (TGF-β) neurodevelopmental signaling pathways (10). Deletion of USP9X in the embryonic forebrain results in defective neural progenitor cell function, and defective cell maturation (14).

TGF-β is a protein that converts cytokines into growth factors. It's a crucial cytokine, especially for the immune system, but it's also been linked to non-immune functions (15). TGF-β binds to TGF receptors I and II, which are serine/threonine kinases that phosphorylate intracellularly (16). The neuroprotective factor TGF-β initiates a signaling cascade that causes phosphorylation of USP9X and subsequent stabilization of ankyrin-G in spines. Interacting with ankyrinG and regulating its stability by USP9X protects dendritic spines (16). TGF-β controls the subsynaptic localization of Ankyrin-G and USP9X. TGF-β has thus been demonstrated to have a significant role in neuronal growth and function. TGF-β could be used as a therapeutic method to repair synaptic deficits in neurodevelopmental diseases (16). USP9X is a critical regulator of the transforming growth factor signaling pathway. In response to TGF stimulation, USP9X controls dendritic formation and neuronal axonal growth (17).

Given the recent literature on the role of synaptic function and protein homeostasis in autism etiology, USP9X, which is one of the main mechanisms of protein homeostatic function, is noted for its role in synaptic function and has not yet been studied in children with OSB. In our study, we aimed to compare levels of USP9X and potentially associated Transformative Growth Factor between children with autism and healthy controls.

MATERIAL AND METHODS

Participants

This is a cross-sectional study aiming to compare serum TGF-β and USP9X levels in children with Autism Spectrum Disorder and healthy controls. Approval for the study was obtained from Gaziantep University Clinical Research Medical Ethics Committee on 12.01.2022 with decision number 2021/347. Patients who met the inclusion criteria among the patients who applied to the Child and Adolescent Psychiatry Outpatient Clinic between January and March 2022 were included in the study. Patients diagnosed with ASD according to DSM-5 criteria and aged between 3–12 years were included in the study. Patients taking any medication other than psychotropic drugs in the last 2 weeks, taking anti-inflammatory and antioxidant agents, having acute or any known chronic metabolic disease, genetic disease, neurological disease, cardiovascular disease, respiratory disease, liver or kidney disease, oncologic disease, hematologic disease, infective disease, autoimmune disease, allergic disease or medical disease were excluded. The control group was selected from people without any known medical or psychiatric illness.

Measurements

Parents of children with ASD and children in the control group signed Informed Voluntary Consent Forms to participate in the study. Literate children over the age of 6 in the control group also gave informed consent. Both groups were evaluated by a child psychiatrist and sociodemographic data were collected from the parents.

Sociodemographic Form: Using a semi-structured interview schedule, the ages and developmental stages of children with ASD and healthy children, the ages and employment status of their parents, and the presence of psychopathology in their parents were questioned.

Childhood Autism Rating Scale (CARS) was filled in by the clinician for children with ASD based on the information obtained both by asking the parents and by observing the child (18). Childhood autism rating scale is a valid and reliable scale widely used in the diagnosis of autism. Childhood autism rating scale is a 15-item scale developed to diagnose autism as well as to distinguish children with developmental disabilities without ASD from children with ASD. The items in the scale are grouped under the headings of relationship with people, imitation, emotional reactions, body use, reaction to change, visual reactions, resting reactions, taste, smell and touch use, fear/nervousness, verbal communication, non-verbal communication, mental activities and general impressions. Childhood autism rating scale is a scale with proven validity and reliability in our country (19).

The Turkish version of the Gilliam Autism Rating Scale-2 (TV-GARS-2) was completed by one of the parents of children with ASD (mother or father; the parent with better knowledge about the child was preferred). The TV-GARS-2 consists of three subscales; Stereotypic Behaviors, Communication and Social Interaction, with items including specific, observable and measurable behaviors. As a result of the evaluation of the TV-GARS-2, a standard score called the Autistic Disorder Index is obtained (20). The Turkish version of GARS-2 is a valid and reliable scale (21).

Collection of Blood Samples and Laboratory Work

Blood was collected from volunteers at the blood collection unit of Gaziantep University Medical Faculty Hospital. The blood samples were placed in a vacuumed plastic gel tube with a yellow tip and centrifuged at 4000 rpm for 10 minutes, after which the serum was separated and stored at -80 degrees Celsius until the analysis period. TGF-β and USP9X levels were determined in the Medical Biochemistry Department of Gaziantep University and analyzed using an ELISA approach with a ready-made kit after the samples were completed.

Statistical Analysis

For categorical variables, frequency and percentage analysis are used as descriptive statistics, whereas mean and standard deviation are provided for numerical variables. Shapiro-Wilk tests were performed on the normal distributions of the USP9X and TGF-β variables. Paired-Student T-test/ Mann-Whitney U test comparison with categorical variables is used to compare these variables. For categorical variables with t wo groups, the

U test was applied, and the Analysis of Variance/Kruskal Wallis test was applied for categorical variables with three or more groups. Results of the study are shown as median (Q1-Q3) for variables that do not follow a normal distribution and as mean and standard deviation for those that do. Chi-square analysis was additionally performed to look at the variations among categorical variables. In addition, the relationships between the numerical variables were examined using Spearman correlation analysis. The analyses were carried out using the IBM Statistical Package for Social Sciences (SPSS) program version 22.0 software. The threshold for significance was fixed at p<0.05.

RESULTS

The sample group consists of a total of 82 individuals, including 41 children with ASD between the ages of 3–12 and 41 healthy children from the same age group. The mean age of the control group was 7.83 (±2.37), and the mean age of the case group was 7.20 (±2.66), and no statistically significant difference was found (p>0.05). When the gender distribution is examined, 65 (79.2%) of 82 children are boys and 17 (19.1%) are girls. The male/female ratio is 4/1. There was no statistically significant difference between the case and control groups when the children were evaluated in terms of gender (p>0.05). No statistically significant difference was found when the two groups were compared according to the presence or absence of psychiatric disease in the mothers and fathers of the participants (p>0.05). When the first degree relatives of the case and control groups were evaluated in terms of psychiatric disease states, a statistically significant difference was found (p<0.05). When the case and control groups were evaluated in terms of the presence of ASD in the family, a statistically significant difference was found (p<0.05). While 1 (2.5%) of the participants in the control group had a family history of ASD, 40 (97.5%) had no family history of ASD. While 10 (24.3%) of the participants in the case group had a family history of ASD, 31 (75.7%) had no family history of ASD. The continuation of the data is given in Table 1 in detail.

According to the scores corresponding to the Autistic Disorder Index in the TV-GARS-2, 1 (2.5%) patient had a low probability of ASD, 3 (7.3%) patients had a high probability of ASD, 9 (21.9%) patients had a high probability of ASD (mild level), 17 (41.4%) patients had a very high probability of ASD (moderate level) and 11 (26.8%) patients had a very high probability of ASD (severe level). According to the CARS scores completed by the clinician, 18 (43.9%) patients met the mild-moderate and 23 (56.1%) patients met the severe ASD scores (Table 2).

As a result of the comparison of USP9X and TGF-β levels between children with ASD and healthy controls, it was found that USP9X level was statistically significantly higher in children with ASD (p=0.001), whereas TGF-β level did not show a significant difference between the two groups (Table 3). In the correlation analysis between the CARS and TV-GARS-2 scores of the cases and USP9X and TGF-β values, no statistically significant difference was found except for the 7th question of the CARS, Visual response subscale. A moderate positive correlation was found between the visual response subscale and USP9X (r=0.337, p=0.031) and TGF-β (r=0.390, p=0.012) (Table 4). According to this result, it was observed that USP9X and TGF-β values increased as the eye contacts of the patients became abnormal.

Table 1. Comparison of participants' sociodemographic information

	Patient	Control	P
Age*	7.20 (±2.66)	7.83 (±2.37)	0.259
Sex**			0.173
Female	6 (14.6%)	11 (26.8%)	
Male	35 (85.4%)	30 (73.2%)	
General developmental steps (speaking, walking, toilet training)***			<0.001
On time	4 (9.8%)	38 (92.7%)	
Delayed	37 (90.2%)	3 (7.3%)	
Maternal psychiatric illness**			0.785
No	33 (80.5%)	32 (78.0%)	
Yes	8 (19.5%)	9 (22.0%)	
Paternal psychiatric illness***			0.131
No	32 (78.0%)	37 (90.2%)	
Yes	9 (22.0%)	4 (9.8%)	
Psychiatric illness in first degree relative**			0.015
No	16 (39%)	27 (65.9%)	
Yes	25 (61%)	14 (34.1%)	
A family history of ASD***			0.004
No	31 (75.6%)	40 (97.6%)	
Yes	10 (24.4%)	1 (2.4%)	
A family history of ADHD**			0.135
No	27 (65.9%)	33 (80.5%)	
Yes	14 (34.1%)	8 (19.5%)	

ADHD: attention deficit hyperactiviy disorder; ASD: autism spectrum disorder; * Independent T-Test; **Chi-square; *** Fisher's test.

Table 2. Autism spectrum disorder severity of cases according to CARS and TV-GARS-2

		n	%
TV-GARS-2 total point Decision guide CARS Point CARS results	ASD* is unlikely to be seen	1	2.4
	ADS is possible	3	7.3
	ASD Possibility Quite High-Light	9	21.9
	ASD Probability Fairly High-Medium	17	41.5
	ASD Possibility Quite High-Heavy Low-medium 32.15 (±6.7) Extremely Autistic 37.6 (±5.9)	11 18 23	26.9 43.9 56.1

ASD: autism spectrum disorder; CARS: childhood autism rating scale; TV-GARS-2:Turkish version of the Gilliam autism rating scale – 2.

Table 3. Comparison of USP9X and TGF-β values between patient and control groups

	Patient	Control	P
USP9X (ng/ml)	11.94 (10.41–13.48)	8.77 (7.27–10.26)	0.001
TGF-β (pg/ml)	466.28 (420.18–512.37)	503.93 (461.48–546.38)	0.209

Mann-Whitney U test.

Table 4. Correlation between cars items and TGF-β and USP9X

		US	TG	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C12	C11	C13	C14	C15	CT
US	r	1	0.162	-0.058	0.137	-0.009	-0.280	-0.139	0.100	0.337*	-0.010	0.120	-0.182	-0.194	-0.083	0.049	-0.218	-0.049	-0.075
	p		0.145	0.721	0.392	0.953	0.076	0.386	0.534	0.031	0.951	0.454	0.254	0.225	0.607	0.761	0.171	0.763	0.639
TG	r	0.162	1	0.009	0.095	-0.042	-0.070	-0.077	-0.051	0.390*	0.117	-0.126	0.207	0.012	0.180	0.085	-0.057	-0.149	0.042
	p	0.145		0.956	0.553	0.796	0.663	0.631	0.752	0.012	0.466	0.434	0.193	0.942	0.261	0.596	0.721	0.354	0.793
C1	r	-0.058	0.009	1	0.490**	0.363*	0.450**	0.542**	0.148	0.182	0.308	0.195	0.171	0.408**	0.523**	0.042	0.345*	0.546**	0.659**
	p	0.721	0.956		0.001	0.020	0.003	0.000	0.354	0.256	0.050	0.221	0.285	0.008	0.000	0.797	0.027	0.000	0.000
C2	r	0.137	0.095	0.490**	1	0.172	0.399**	0.556**	0.126	0.494**	0.515**	0.303	-0.037	0.274	0.539**	-0.258	0.608**	0.617**	0.665**
	p	0.392	0.553	0.001		0.283	0.010	0.000	0.433	0.001	0.001	0.054	0.818	0.083	0.000	0.103	0.000	0.000	0.000
C3	r	-0.009	-0.042	0.363*	0.172	1	0.350*	0.620**	0.426**	0.140	0.140	0.523**	0.315*	0.222	0.235	0.262	0.099	0.463**	0.628**
	p	0.953	0.796	0.020	0.283		0.025	0.000	0.005	0.382	0.384	0.000	0.045	0.163	0.139	0.098	0.537	0.002	0.000
C4	r	-0.280	-0.070	0.450**	0.399**	0.350*	1	0.456**	0.028	0.123	0.232	0.300	0.067	0.368*	0.404**	0.003	0.343*	0.539**	0.606**
	p	0.076	0.663	0.003	0.010	0.025		0.003	0.861	0.445	0.145	0.057	0.677	0.018	0.009	0.986	0.028	0.000	0.000
C5	r	-0.139	-0.077	0.542**	0.556**	0.620**	0.456**	1	0.230	0.278	0.275	0.469**	0.102	0.498**	0.588**	0.117	0.448**	0.707**	0.821**
	p	0.386	0.631	0.000	0.000	0.000	0.003		0.147	0.079	0.082	0.002	0.526	0.001	0.000	0.466	0.003	0.000	0.000
C6	r	0.100	-0.051	0.148	0.126	0.426**	0.028	0.230	1	0.101	0.087	0.291	0.271	-0.089	0.054	0.175	0.041	0.323*	0.378*
	p	0.534	0.752	0.354	0.433	0.005	0.861	0.147		0.530	0.589	0.065	0.087	0.580	0.738	0.272	0.798	0.039	0.015
C7	r	0.337*	0.390*	0.182	0.494**	0.140	0.123	0.278	0.101	1	0.694**	0.097	-0.126	0.199	0.315*	-0.076	0.141	0.406**	0.442**
	p	0.031	0.012	0.256	0.001	0.382	0.445	0.079	0.530		0.000	0.548	0.432	0.212	0.045	0.635	0.379	0.009	0.004
C8	r	-0.010	0.117	0.308	0.515**	0.140	0.232	0.275	0.087	0.694**	1	0.023	-0.035	0.191	0.415**	-0.213	0.258	0.534**	0.502**
	p	0.951	0.466	0.050	0.001	0.384	0.145	0.082	0.589	0.000		0.886	0.829	0.231	0.007	0.180	0.104	0.000	0.001
C9	r	0.120	-0.126	0.195	0.303	0.523**	0.300	0.469**	0.291	0.097	0.023	1	0.294	-0.029	0.140	-0.094	0.409**	0.425**	0.512**
	p	0.454	0.434	0.221	0.054	0.000	0.057	0.002	0.065	0.548	0.886		0.062	0.859	0.384	0.560	0.008	0.006	0.001
C10	r	-0.182	0.207	0.171	-0.037	0.315*	0.067	0.102	0.271	-0.126	-0.035	0.294	1	0.031	0.147	0.091	0.188	0.098	0.288
	p	0.254	0.193	0.285	0.818	0.045	0.677	0.526	0.087	0.432	0.829	0.062		0.846	0.360	0.571	0.240	0.540	0.067
C12	r	-0.194	0.012	0.408**	0.274	0.222	0.368*	0.498**	-0.089	0.199	0.191	-0.029	0.031	1	0.561**	0.053	0.394*	0.520**	0.546**
	p	0.225	0.942	0.008	0.083	0.163	0.018	0.001	0.580	0.212	0.231	0.859	0.846		0.000	0.744	0.011	0.000	0.000
C11	r	-0.083	0.180	0.523**	0.539**	0.235	0.404**	0.588**	0.054	0.315*	0.415**	0.140	0.147	0.561**	1	0.056	0.609**	0.688**	0.734**
	p	0.607	0.261	0.000	0.000	0.139	0.009	0.000	0.738	0.045	0.007	0.384	0.360	0.000		0.729	0.000	0.000	0.000
C13	r	0.049	0.085	0.042	-0.258	0.262	0.003	0.117	0.175	-0.076	-0.213	-0.094	0.091	0.053	0.056	1	-0.230	-0.014	0.161
	p	0.761	0.596	0.797	0.103	0.098	0.986	0.466	0.272	0.635	0.180	0.560	0.571	0.744	0.729		0.147	0.929	0.316
C14	r	-0.218	-0.057	0.345*	0.608**	0.099	0.343*	0.448**	0.041	0.141	0.258	0.409**	0.188	0.394*	0.609**	-0.230	1	0.645**	0.615**
	p	0.171	0.721	0.027	0.000	0.537	0.028	0.003	0.798	0.379	0.104	0.008	0.240	0.011	0.000	0.147		0.000	0.000
C15	r	-0.049	-0.149	0.546**	0.617**	0.463**	0.539**	0.707**	0.323*	0.406**	0.534**	0.425**	0.098	0.520**	0.688**	-0.014	0.645**	1	0.888**
	p	0.763	0.354	0.000	0.000	0.002	0.000	0.000	0.039	0.009	0.000	0.006	0.540	0.000	0.000	0.929	0.000		0.000
CT	r	-0.075	0.042	0.659**	0.665**	0.628**	0.606**	0.821**	0.378*	0.442**	0.502**	0.512**	0.288	0.546**	0.734**	0.161	0.615**	0.888**	1
	p	0.639	0.793	0.000	0.000	0.000	0.000	0.000	0.015	0.004	0.001	0.001	0.067	0.000	0.000	0.316	0.000	0.000	

C: CARS; CT: total score of CARS; TG: TGF-β; US: USP9X.
Spearman correlation test.

DISCUSSION

In our study, when USP9X and TGF-β levels were compared between the case and control groups, USP9X levels were significantly higher in the case group, whereas no significant difference was found between the groups in terms of TGF-β levels. In our study, we also discovered a link between decreased eye contact and increased USP9X and TGF-β levels.

USP9X is a potential gene for human neurodevelopmental disorders including lissencephaly, epilepsy, X-linked intellectual disability, and autism spectrum disorders (22). When looking at the literature, attention has been drawn to TGF-β and USP9X as potential players in understanding the complex processes that cause autism, but there seems to be little work in this area.

Studies in mice in this area have played a central role in expanding our understanding. In an animal study, for example, the most prominent phenotype of USP9X-deficient mice was the dramatic reduction in the size of the adult hippocampus. In this study, it was reported that in the absence of USP9X, the overall architecture of the brain develops normally, but the corpus callosum and hippocampal dimensions are reduced. The researchers also noted that the absence of USP9X leads to dramatic reductions in axonal length and attributed this to a failure in TGF-β signaling (10). In an animal study where forebrain-specific USP9X knockout mice (USP9X -/y) were analyzed, researchers noted that USP9X -/y mice exhibited abnormal communication and social interaction behaviors. In contrast, they noted that no repetitive behavior was observed in USP9X -/y mice (23).

In another animal study, mice lacking USP9X showed a dramatic reduction in dentate gyrus size and were found to have a smaller hippocampus. When cellular populations in the dentate gyrus of USP9X-deficient mice were analyzed in this study, abnormal neuroblast morphology with reduced stem cells and reduced neuroblasts were detected (24). These studies emphasize the critical role USP9X plays in the normal morphological development of the postnatal hippocampus. Studies have shown that hippocampal activity is impaired in individuals with ASD. The hippocampus plays critical roles in social interaction, memory and spatial reasoning, which are impaired in autism (25). Considering these findings, we think that the relationship between USP9X and autism is an important subject of study, but when we look at the literature, we see that there is a big information gap in this field. Although the relationship between USP9X and intellectual disability has been partially studied, the relationship between USP9X and autism is still unclear.

Mutations in USP9X are thought to cause neurodevelopmental problems in mammals, including humans. Intellectual disability, autism, epilepsy, and lissencephaly have all been linked to the loss of USP9X function (24).

In a review of the literature, USP9X has been associated with neurodevelopmental problems, including developmental delay/intellectual disability in both men and women, with a variety of inheritance patterns and clinical manifestations, consistent with its role during brain development (14,26). A report on a truncating mutation in USP9X that correlates with disease in a family with X-linked mental retardation has been published (27). In a study involving a total of 5 individuals with USP9X mutations, intellectual disability was found in all 5 individuals and autism with intellectual disability was found in 2 of 5 individuals. Mutations in USP9X have been reported to have a functional effect and cause intellectual disability (22).

De Laurentiis et al. (2023) reported a 5-year-old boy with a classic neurodevelopmental phenotype who had a missense variant in USP9X. This case, which presented with a previously unreported radiological picture of periventricular heterotopia, highlighted the critical role of USP9X in cortical architecture and organization of neural migration and the existence of an X-linked form of USP9X neurodevelopmental disease (28). Whole-exome sequencing was performed to identify risk genes/rare variants in a cohort of 19 cases with autism and 19 triplet groups including both parents of these cases, and it was reported that the USP9X gene, along with many other genes, may be associated with autism (29).

Animal and very limited human studies on USP9X suggest an association between USP9X mutation and autism. In contrast to the literature, USP9X levels in our study were higher in individuals with autism compared to the control group, which may be related to our small sample group, limiting our ability to detect differences. Given that studies have shown that intellectual disability is often associated with USP9X loss-of-function mutations, the fact that the relationship between the degree of intellectual disability and USP9X was not examined in this study is also an important limitation of our study. In addition, the fact that USP9X has different results according to gender seems to make it difficult for us to interpret the results. We know that USP9X is located on the X chromosome. The inheritance patterns and clinical presentation of X-linked disorders often differ between men and women. X-linked disorders predominantly affect hemizygous males, while female carriers are usually unaffected (30). Protection of heterozygous females in X-linked disorders may involve protective X inactivation. However, USP9X is an atypical X chromosome gene that escapes X inactivation, meaning it is expressed from both active and inactive chromosomes. The degree of escape is reported to vary between genes, tissues, and individuals, possibly contributing to phenotypic heterogeneity (26,30). For genes such as USP9X that escape X chromosome inactivation, the neurodevelopmental disorder phenotype can be lethal in males (31). The fact that USP9X measurement in our study was based on peripheral blood levels suggests that serum levels may not correlate with brain levels for genes that escape X chromosome inactivation and that serum levels are not a useful marker for genes that escape X chromosome inactivation.

In a study using patient-derived cell lines, data showed that USP9X variants were involved in a marked neurodevelopmental and behavioral syndrome in male subjects. This study points to loss of TGF-β signaling and hippocampal function as major contributors to pathology (14).

TGF-β signaling is lost in USP9X missense variants. In neurodevelopmental signaling pathways, this results in a reduction in substrate levels. While the USP9X missense mutation inhibits TGF-β mediated axonogenesis, it causes mTOR-mediated decreased neural stem cell proliferation and notch signaling disruption with WNT-mediated neural proliferation and differentiation. Axonogenesis is inhibited by TGF-β, resulting in agenesis of the corpus callosum, enlarged ventricles and various brain abnormalities. This results in hypotonia, motor defects and visual defects, as well as decreased grip strength, body tone, gait and visual position (14).

Considering the loss of USP9X substrates and the combined defect of TGF-β signaling in neurodevelopmental disorders, both USP9X and TGF-β levels were measured in our study. Several studies have shown that TGF-β1 levels are reduced in the serum and cerebrospinal fluid of children with autism. A 2007 study by Kyoko et al. suggested that there may be a link between low TGF-β levels and autism (32). In a study comparing 44 children with autism and 45 healthy controls, decreased TGF-β and IL-10 production was associated with autism (33). In another study with a similar sample size, no association was found between TGF-β1 codon 10 and ASD, whereas a significant association was found between TGF-β1 codon 25 and ASD development (34). Vargas et al. reported increased TGF-β2 levels in children with autism (35). According to the findings of a study conducted in Egypt, it was reported that there

may be a link between the severity of autism symptoms and TGF-β levels. They reported that behavioral symptoms such as stereotypy, irritability and hyperactivity worsened as TGF-β levels decreased (36). In another study, a correlation was found between lower TGF-β and more violent behavior scores in children with ASD. In addition, a positive correlation was found between TGF-β1 concentration and autism severity, and a negative correlation between TGF-β2 and autism severity (37).

TGF-β has at least 3 isoforms (TGF-β1, TGF-β2 and TGF-β3). TGF-β1 has an important regulatory role in the development of the central nervous system and has potential implications for neurogenesis in various central nervous system diseases (37). Some researchers have also found that TGF-β1 plays a suppressive role in early central nervous system development and has an important role in neuronal migration, survival, and synapse formation (38).

Although the study found no significant difference between the case and control groups in terms of TGF-β levels, the TGF-β level of the case group was found to be lower. Low TGF-β levels in the autism group support the literature. The fact that TGF-β subtypes were not measured separately in our study may have limited our findings. It also remains unclear whether serum levels of TGF reflect those in the brain (39). The absence of a statistically significant difference may also be the result of the curative effect of the treatment. Because it is stated that treatment has positive effects on cytokine levels and symptom severity (40,41). A study that included 55 drug-naive first-episode psychosis patients and 57 healthy controls noted that risperidone significantly normalized the initially abnormal cytokine profiles in first-episode psychosis patients (40). Studies investigating the relationship between antidepressant drugs and oxidative stress have shown that antidepressants reduce oxidative stress by strengthening antioxidant defense (41). Irritability, hyperactivity, stereotyped behavior, social disengagement, and incorrect speech have all been found to improve when risperidone is used with pentoxifylline, a proinflammatory cytokine inhibitor and immunomodulatory medication (42). Furthermore, when combined with the nonsteroidal anti-inflammatory medicine celecoxib, risperidone has been shown to generate significant regressions in the irritability, social disengagement, and stereotypy subscales of autism (43).

The present study we found that USP9X and TGF-β levels correlated with the eye contact subscale. Restricted eye contact is one of the red flag signs of ASD and it is well known that eye contact can be compromised even in moderate cases. Although we did not discover any link between USP9X and ASD severity in our study, the positive correlation between USP9X and eye contact suggests the possibility of a potential link between USP9X and autism. To understand whether such a link exists, the relationship between USP9X and Autism spectrum disorder needs to be examined in a larger sample and in a more comprehensively designed study.

The study has some limitations that should be taken into account. These include the cross-sectional nature of the study, the small sample size, the use of psychotropic drugs in the patient group, which may affect the levels of biomarkers, and the measurement of biological parameters from peripheral blood. The strengths of this study are that it is the first study to measure USP9X levels, which has been emphasized as a potential player in understanding the complex processes that cause autism, the exclusion of genetic or chronic medical diseases, and the administration of the CARS scale by the same clinician. We also adopted a strict matching procedure to reduce the potential confounding effects of demographic characteristics such as age and gender on the study results.

In conclusion, there may be an association between USP9X and autism, but measuring USP9X levels in serum limits the results of the study. Future studies in a larger population and including combinations of different biomarkers in the same pathway will provide a better understanding of the pathophysiology of ASD and guide possible treatments.

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